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نموذج رقم (١٨)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية
وقوانين الجامعة الأردنية وأنظمتها وتعليماتها
لطلبة الماجستير

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تخصص: العلوم الطبية المخبرية   الكليـــــة: الطب

عنوان الرسالة: ....
In vitro selection of anti Platelet Derived Growth Factor Receptor aptamers by Systematic Evolution of ligand by Exponential Enrichment (SELEX).

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد رسائل الماجستير عندما قمت شخصيا" باعداد رسالتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الرسائل العلمية. كما أنني أعلن بأن رسالتي هذه غير منقولة أو مستلة من رسائل أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

توقيع الطالب: ....   التاريخ: ١٥ / ٥ / ٢٠١١م

IN VITRO SELECTION OF ANTI- PLATELET DERIVED GROWTH FACTOR RECEPTOR APTAMERS BY SYSTEMATIC EVOLUTION OF LIGAND BY EXPONENTIAL ENRICHMENT (SELEX)

By
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This thesis was Submitted in Partial Fulfillment of the Requirements for the Master's Degree of Science in Medical Laboratory Sciences

Faculty of Graduate Studies
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May, 2011

COMMITTEE DECISSION

This thesis (IN VITRO SELECTION OF ANTI- PLATELET DERIVED GROWTH FACTOR RECEPTOR APTAMERS BY SYSTEMATIC EVOLUTION OF LIGAND BY EXPONENTIAL ENRICHMENT (SELEX)) was successfully defended and approved on 9/5/2011.

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DEDICATION

To

My father and mother for their encouragement & continuous support to overcome all difficulties, for making sacrifices and for understanding that this was something I wanted to do

My dearest & beloved family, thank you for being the light of my life

To

My best friends for everything

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Finally, thanks to everyone who aided in the completion of this study.

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LIST OF ABBREVIATION

ALL	**Acute Lymphoolastic Leukemia.**
AML	**Acute Myeloid Leukemia.**
ATP	**Adenosine Tri-Phosphate.**
AMD	**Age related Macular Degeneration.**
bp	**Base pair.**
CE	**Capillary Electrophoresis.**
CML	**Chronic Myelocytic Leukemia.**
CMML	**Chronic Myelomonocytic Leukemia.**
C5	**Complement 5.**
CDK	**Cycline Dependent Kinase.**
dNTPs	**Deoxynucleotide triphosphate.**
DNA	**Deoxyribonucleic Acid.**
DMSO	**Di-Methyl Sulphoxide**
Kd	**Dissociation of constant.**
ELISA	**Enzyme Linked ImmunoSorbent Assay.**
ELONA	**Enzyme Linked Oligonucleotide Assay.**
EGFR	**Epithelial growth factot receptor.**
EDTA	**Ethylenediaminetetraacetic acid**
FGFR	**Fibroblast Growth Factor Receptor**
FLT-3	**Fms- like Tyrosine 3.**
FDA	**Food and Drug Administration.**
GQRS	**Guanine Quadraplex Rich Sequence.**
GRO	**Guanine Rich Oligonucleotide.**
HCV	**Hepatitis C Virus.**
HIV	**Human deficiency viruses.**
HES	**Hypereosinophilic Syndrome.**
IgE	**Immunoglobulin E.**
IPTG	**Isopropyl-1-thio-β-D-Galactoside**
JAK-2	**Janus Kinase 2.**
JM	**juxtamembrane domain.**
KDa	**Kilo Dalton**
KI	**Kinase Insert domain.**
LB media	**Luira Broth Media**
MALDI-MS	**Matrix Assisted Laser Desorption / Ionization – Mass Spectrometer.**
MBs	**Methylene Blue.**
NPs	**Nanoparticles.**
NRTK	**Non Receptor Tyrosine Kinases**
NS3	**Non Structural protein 3.**
PI3K	**Phosphatidyl Inositol 3 Kinase.**
PLC γ	**Phospholipase C γ.**
PDGFRα	**Platelet-Derived Growth Factor Receptor alpha.**
PDGFR β	**Platelet-Derived Growth Factor Receptor beta.**
PDGF	**Platelet-Derived Growth Factor.**
PCR	**Polymerase Chain Reaction.**
PSMA	**Prostate Specific Membrane Antigen.**
RTK	**Receptor Tyrosine Kinase.**

RT	**Reverse Transcription**
ssDNA	**Single stranded DNA.**
siRNA	**Small interference RNA.**
SH2	**Src Homology 2.**
SELEX	**Systematic Evolution of Ligand by Exponential Enrichment**
TAR	**Trans Activation Response.**
TM	**Transmembrane domain.**
TBE	**Tris Borate EDTA.**
VEGF	**Vascular Endothelial Growth Factor.**
X - gal	**5-Bromo-4-chloro-3-indolyl-β-D galactoside**

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IN VITRO SELECTION OF ANTI- PLATELET DERIVED GROWTH FACTOR RECEPTOR APTAMERS BY SYSTEMATIC EVOLUTION OF LIGAND BY EXPONENTIAL ENRICHMENT (SELEX)

By
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ABSTRACT

Background: Aptamers are nucleic acids which are isolated from combinatorial oligonucleotide libraries by an in vitro selection process called Systematic Evolution of Ligand by Exponential Enrichment (SELEX). Aptamers can fold into three dimensional shapes and structures, and are able to bind to different molecules including proteins with high affinity and specificity comparable to monoclonal antibodies.
Aptamers could modulate the activity of target proteins through its binding to a specific region. One of these proteins is PDGFR which is a receptor tyrosine kinase that consists of two distinct polypeptide subunits; α and β. Both are known to play key roles in many cancers when acquired genetic abnormalities lead to constitutive activation of PDGFR.

Aims of the study: This study aims to develop nucleic acid ligands (Aptamers) capable of binding with the kinase domain of PDGFRα and PDGFRβ, and the subsequent analysis of the aptamer sequences.

Methods: The starting oligonucleotide library consisted of 90 bases in length with a central region comprising 45 random nucleotides. This library was incubated with Histidine tagged PDGFRα and PDGFRβ, where the molecules that do not bind to the targets were washed away, and the remainder were harvested and amplified by polymerase chain reaction (PCR). The aptamer-enriched pool of single stranded molecules was then isolated from the PCR product, reapplied to the PDGFRs and the process was repeated to select the sequences that bind to the targets with high affinity and specificity.

Results: Following the tenth round of selection; the enriched aptamer pool was cloned and 24 clones were sequenced; 10 for PDGFRα and 14 for PDGFRβ. The selected aptamer sequences were analyzed to determine the conserved sequence that can play an important role in the inhibitory effect of the aptamers, and compare the selected aptamers with sequences already known to be tyrosine kinase inhibitors.

Conclusions: this study has described the process for selection of anti- PDGFRα/β aptamers. The properties of the selected aptamers make these molecules appealing candidates for further development as enzymatic inhibitors both for laboratory use and as therapeutic agents in the future.

1. Introduction.

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) is a screening technique that involves iterative cycles of selection of nucleic acid ligands which interact with the target in a desirable manner and amplification of the nucleic acid starting from a large library of oligonucleotides with different sequences (Sampson, 2003; Klug and Famulok, 1994). The products of the selection are called aptamers, which are short single stranded DNA, RNA, or dsDNA, or molecules with three dimensional structures, which bind with high affinity and specificity to a large variety of targets, ranging from small molecules to complex mixtures (Wilson and Szostak, 1999). Aptamers are comparable to monoclonal antibodies in their mechanism of action, expressing high specificity and high affinity (Gold et al., 1995; James, 2000). It was initially introduced by two groups of workers and it is based on general nucleic acid and protein separation techniques (Tuerk and Gold, 1990; Ellington and Szostak, 1990). Aptamers are widely used in many applications, being analytical, diagnostic or therapeutic.

Tyrosine kinases are enzymes that phosphorylate tyrosine residues by transforming the γ phosphate of ATP to tyrosine residues on protein substrates, on the kinase itself (autophosphorylation), or downstream signaling proteins (Till and HubBard, 2004). They play crucial roles in hematopoiesis, hematopoietic stem cell functions and hematologic malignancies.

Platelet- derived growth factor receptor (PDGFR) α and β is a member of type III tyrosine kinase receptors that are characterized by the presence of three main parts; an extracellullar ligand binding domain, single transmembrane hydrophobic domain and the intracellular cytoplasmic kinase domain (Funa et al., 2003; Swick et al., 2008). Because of the critical role of these receptors on the regulation of cell growth, abnormalities in these proteins can lead to constitutive activation of PDGFRs that may have pathological

consequences. Accordingly, tyrosine kinases, including PDGFR, are considered as potential targets for many newly developed anti-cancer drugs and therapies. Selection of anti-PDGFRα and anti-PDGFRβ inhibitor aptamers will hopefully be effective in the treatment of many disorders caused by up regulation of PDGFRs (Stover et al., 2005; Dova et al., 2007).

2. Literature review

2.1 Nucleic acids.

The discovery of deoxyribonucleotide structure (DNA) is considered as a major achievement of the twentieth century (Watson and Crick, 1953). For a long time, nucleic acids were considered mainly as linear carriers of information. In addition to their templating functions, nucleic acids could fold into complex structures and perform various other biological activities. It was found that single stranded RNA molecules are able to form very different three dimensional structures, which allow them to recognize specifically molecular targets and even perform catalysis. As an example, tRNA and rRNA molecules play a key role in protein synthesis, and the *Tetrahymena thermophilia* group 1 intron as a naturally occurring RNA that catalyzes a transesterification reaction (Been and Cech, 1988; Brugert et al., 2002). Other examples emerged from studies on Human Immunodeficiency Virus (HIV) and Adenoviruses which showed the ability of these viruses to encode small structural nucleic acid ligands that bind to viral or cellular proteins with high affinity and specificity and modulate the activity of these proteins that are essential for the replication of these viruses or inhibit the activity of cellular proteins by working as anti-viral agents (Marciniak et al., 1990; Brugert et al., 2002).

The combinatorial variety of nucleic acids and their ability to form diverse secondary and tertiary structures make it possible to select RNA and ssDNA molecules capable of recognition of other molecules and exhibiting catalytic activity (Pendergrast et al., 2005).

2.2 Combinatorial chemistry

Combinatorial chemistry is an important technology for industry as well as biotechnological and pharmaceutical research to discover new materials or molecules with desirable properties, new drugs, and catalysts. It is characterized by the synthesis and simultaneous screening of large libraries of related, but structurally distinct compounds to identify and isolate functional molecules. Nucleic acids are very attractive compounds for combinatorial chemistry, since they are able to fold into defined secondary and tertiary structures, and they can be amplified by PCR or in vitro transcription easily. Very complex libraries of random sequence oligonucleotides with about 10^{15} different molecules can be produced by chemical synthesis and screened in parallel for a particular functionality, such as high-affinity ligand-binding (aptamers) or catalytic activity (ribozymes) (Verdine 1996; Kopylov and Spiridionova, 2000; David et al., 2004).

2.3 Aptamers and SELEX.

2.3.1 The concept of aptamer

The term aptamers is derived from the Latin word "aptus" which means fitting and the Greek word "meros" meaning particle (Ellington and Szostak, 1990). They can be ssDNA, dsDNA, RNA or peptide molecules that are generated by in vitro selection from large populations of random sequence libraries that have the ability to fold into three dimensional structures produced by a combination of Watson-crick base pairing and non-canonical intermolecular interactions. Aptamers show very high specificity enabling them to distinguish between protein isoforms and different conformational forms of the same protein (Sciwert et al., 2000).

Aptamer also have a high affinity for their targets, with dissociation constants typically from the micromolar to low picomolar range, which is comparable to those of some monoclonal antibodies and sometimes even better (Jayasena, 1999; James, 2007; Famulok et al., 2007).

2.3.2 History

The process of aptamer generation emerged from pioneering experiments by two independent groups in 1990. Tuerk and Gold (1990) described a process of in vitro selection, duped "SELEX" (Systematic Evolution of Ligands by Exponential Enrichment) to determine the sequence requirements of bacterial T4 DNA polymerase, in which the library was based on the natural target hairpin structure with an eight base region of random RNA. Shortly thereafter, Ellington and Szostak, (1990) who originated the term "aptamer" to describe these nucleic acid ligands, published their experiments utilizing in vitro selection to isolate nucleic acids with specific ligand binding properties. Starting nucleic acid libraries were comprised of molecules containing 100 nucleotides of randomized sequences, affinity chromatography was used to isolate RNAs that demonstrated highly specific binding to several organic dyes, none of which had been known to bind to the nucleic acid (Ellington and Szostak, 1990; Tuerk and Gold, 1990).
Since 1990, aptamers have been generated against hundreds of molecular targets, ranging from small molecules to large proteins.

2.3.3 Structural features of aptamers.

A distinguishing feature of aptamers is their ability to form secondary structures. The secondary structure of single-stranded nucleic acids is built from substructures that are assumed to contribute additively to the free energy of the molecules (Santalucia, 1998). There are many structural motifs that aptamers can form, such as hairpin structures,

psudokonots, G- quartet, and G- quadruplex structures (Tuerk et al., 1992; Kulbachinskiy, 2007).

The quadruplex structures formed by guanine-rich nucleic acid sequences have received significant attention because of growing evidence for their role in important biological processes and as therapeutic targets (Sharma, S, et al., 2005; Kelland, L.R., 2005).

The G- quadruplex structure also known as a G-quartet, is formed by repeated folding of either the single polynucleotide or by association of two or four molecules. The structure consists of stacked G-tetrads, which are square co-planner arrays of four guanine bases each. G-quadruplex is stabilized with cyclic Hoogsteen hydrogen bonding between the four guanines within each tetrad (Simonsson, T., et al., 1998; Simonsson, T., 2001).

DNA as a molecule in solution is more stable than RNA and less easily degraded. However, DNA secondary structures are less stable because of weaker staking energies (Santalucia and Hicks, 2004; Flamm et al., 2000). Stable structures of aptamers allow them to bind to their targets via van Der waals, hydrogen bonding, and electrostatic interactions (Pendergrast et al., 2005).

2.3.4 SELEX.

Systematic evolution of ligands by exponential Enrichment **(**SELEX) is a screening technique that involves in-vitro selection and evolution of nucleic acid ligands from large combinatorial nucleic acid libraries that have the potential to bind in high affinity and specificity to a desired target (James, 2000; Gopenath, 2006). Each selection round consists of three stages: (I) incubation of a random library, containing a large number of sequences capable of formation of different structures with targets, (II) separation of

bound and unbound oligonucleotides, (III) amplification of target bound oligonucleotides (Figure 1). The enriched library is used for the next round of selection (Sampson, 2003).

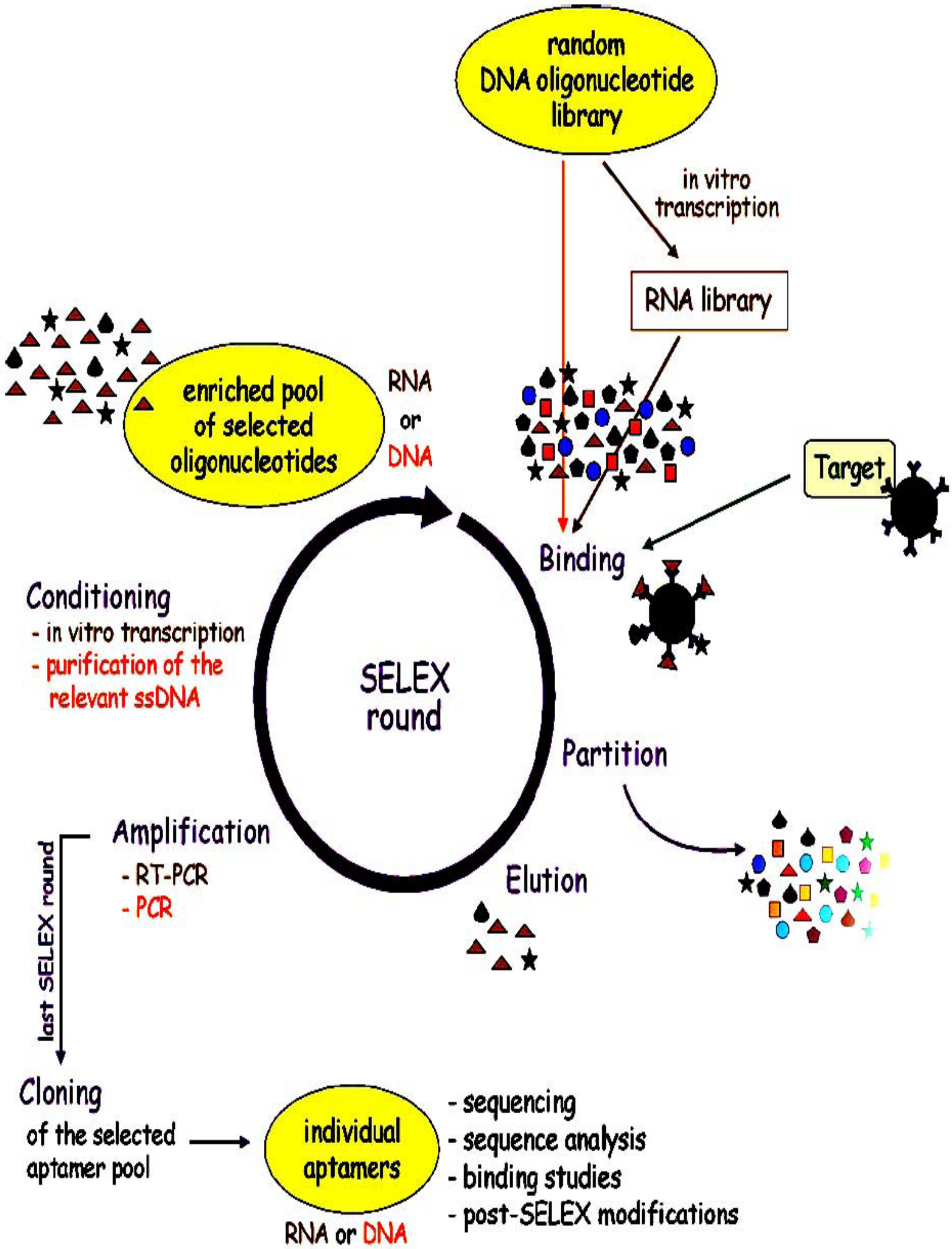


Figure 1. SELEX Protocol. (Stoltenburg et al., 2007).

The number of rounds of selection necessary is determined by both the type of library used as well as by the specific enrichment achieved per selection cycle. On average, enrichment is reached within 5-15 cycles (Cerchia et al., 2002). After completion of selection, the enriched library is cloned and sequences of individual aptamers are determined (Cassiday and Maher, 2003; Wange Wei et al., 2009).

For the selection of RNA aptamers, the random DNA oligonucleotide library has to be transformed into an RNA library before starting the first round of SELEX. A special sense primer with an extension at the 5'- end containing the T7 promoter sequence and an antisense primer are necessary to convert the ssDNA library into a dsDNA library by PCR. The dsDNA is then in vitro transcribed by the T7 RNA polymerase resulting in a randomized RNA library, which is used to start an RNA SELEX. During SELEX process the selected RNA of each round has to be reverse transcribed and subsequently amplified by PCR using the same primers. The new RNA pool for the next SELEX round is then generated again by in vitro transcription (Marshall and Ellington, 2000; Ulrich et al., 2001).

2.3.5 Types of SELEX

Various improvements of the original SELEX method described in 1990 have been reported during the recent decade such as cell SELEX, genomic SELEX, toggle SELEX, photo SELEX and others. These new variants generally shorten the time of selection and improve aptamer affinity and specificity (Yang et al., 2007).

2.3.5.1 Negative SELEX.

Negative SELEX is a screening method used to exclude absorbed ssDNA or RNA by the matrixes used for immobilizing targets with the purpose of getting aptamers that can only bind to targets and to increase the affinity of selected aptamers. This method was put

forward by Ellington and Szostak first in 1990. They used it after the third cycle to eliminate the sequences that could bind to the agarose matrix. Compared with the strategy without negative selex, the affinity of the ssDNA gained by the forth cycle with negative selex was about ten times higher (Ellington and Szostak, 1992; Yang, 2007).

2.3.5.2 Counter SELEX.

Counter SELEX is a unique type of SELEX that is established to get aptamers with stronger specificity. The aptamers selectivity is improved by excluding ssDNA or RNA molecules with coaffinity to the similar molecules of the target. This method was established by Jenison and coworkers when they were identifying RNA aptamers for bronchodilator theophylline. They used caffeine which differs from theophylline only by a methyl group as the counter target (Figure 2) and finally obtained the aptamer which can bind to theophylline only (Jenison et al., 1994).

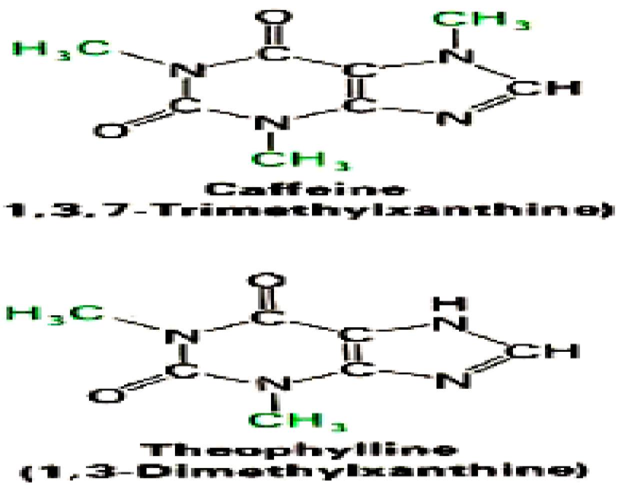


Figure 2. Chemical structure of Theophylline and Caffeine (Jenison et al., 1994).

2.3.5.3 Photo SELEX.

Photo SELEX is a screening method that uses the photosensitive nucleic acids that are able to form a covalent cross-link with other molecules under certain wavelengths of

light. The oligonucleotides are modified by functional groups that can be activated upon irradiation. This method was first used by Jensen who obtained aptamers for the Rev gene (Regular of vision protein expression) of HIV-1 virus (Jensen et al., 1995).

The use of photo-aptamers significantly enhances specificity of protein target recognition because the cross link formation requires correct mutual arrangement of the nucleic acid and protein sites involved in the cross link (Koch et al., 2004).

2.3.5.4 Mirror image SELEX

When aptamers are used in medical treatment, the improvement of their stability in vivo becomes the key point. Furthermore, stable aptamers can also be obtained by mirror image selex. Since RNA and DNA polymerases are not able to carry out polymerization of L- nucleotide, selection in this case is performed by a standard method. After determination of a primary structure of obtained aptamers, the oligonucleotide L-variant is synthesized according to the law of symmetry. These molecules are termed "spiegelmers" from the German "Spiegel" or mirror that consist of L-nucleotides which are characterized by very high stability as they are not recognized by natural nucleases (Eulberg et al., 2003; Faulhammer et al., 2004). Being nuclease resistant, inducing minimal immunogenic response and non-toxic, spiegelmers are ideal candidates for in vivo and in vitro diagnostics; RNA- based and DNA- based spiegelmers have been studied for their applications as in vivo imaging agents (Mairal, et al., 2008).

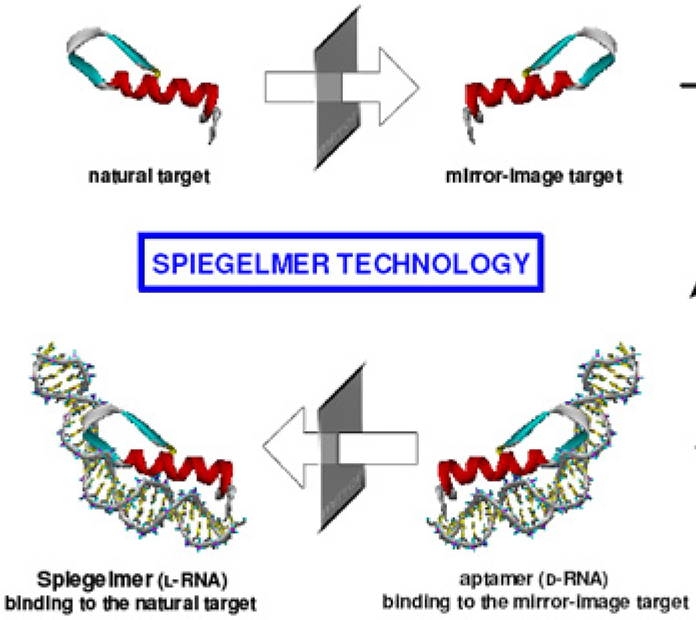


Figure 3. Spiegelmer technology (Mairal, et al., 2008).

2.3.5.5 Cell SELEX

Cell based aptamer selection have been developed as a strategy to use the differences at the molecular level between any two types of cells for the identification of molecular signatures on the surface of targeted cells. The library is incubated with the target cells to allow binding to take place. The cells are then washed and the DNA sequences bound to the cell surface are eluted. The collected sequences are then allowed to interact with excess negative control cells. Only the DNA sequences remaining free in the supernatant are collected and amplified for the next round of selection. After multirounds of selection, the subtraction process efficiently reduced the DNA sequences that bound to the control cells, while those target-cell specific aptamer candidates are enriched (Figure 4).

This method is widely used in aptamer screening for cancer cells, and the aptamers gained have potential usages in the examining and therapy of tumors (Shangguan et al., 2006; Phillips et al., 2008).

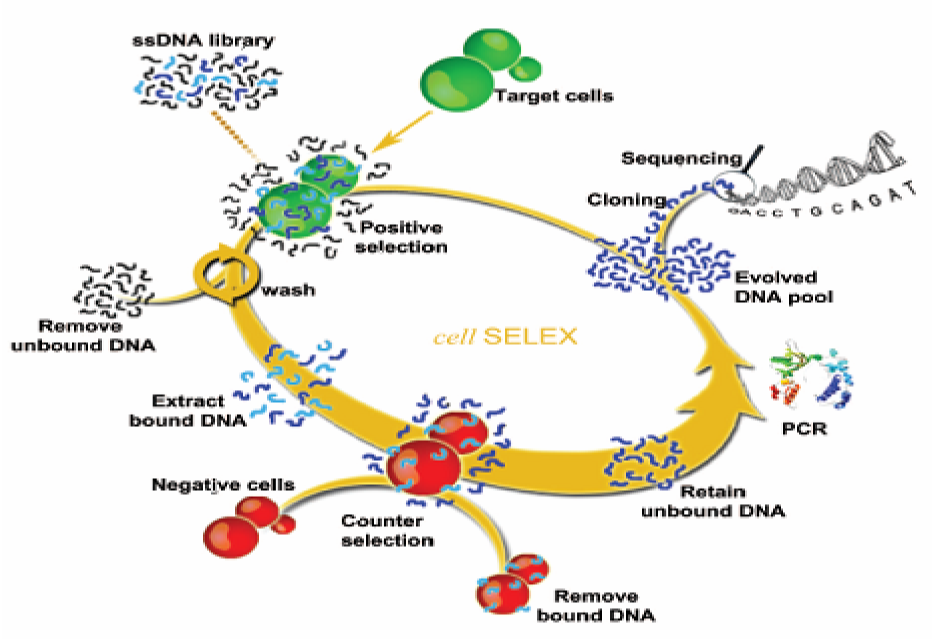


Figure 4. Cell SELEX procedure (Shangguan et al., 2006).

2.3.5.6 Toggle SELEX.

The intricate nature of the protein aptamer interaction results in the production of aptamers which are highly specific for the target protein, but in some types, the species cross reactivity is desired in the pre-clinical evaluation of a molecule in animal models. Therefore, in vitro selection strategies that facilitate the generation of therapeutic agents that are cross reactive between species have been developed. The selection process was named as toggle selex and allowed isolation of aptamers with a broad range of specificities by selecting against alternating cycles (Figure 5). RNA aptamers that bind both human and porcine thrombin were selected by this method; they used both human thrombin and porcine thrombin as targets in the first round of selection and then used human thrombin in the following even rounds of selection and porcine thrombin in the

odd rounds of selection until proper sequences were gained to achieve aptamers with different level of specificity (White et al., 2001).

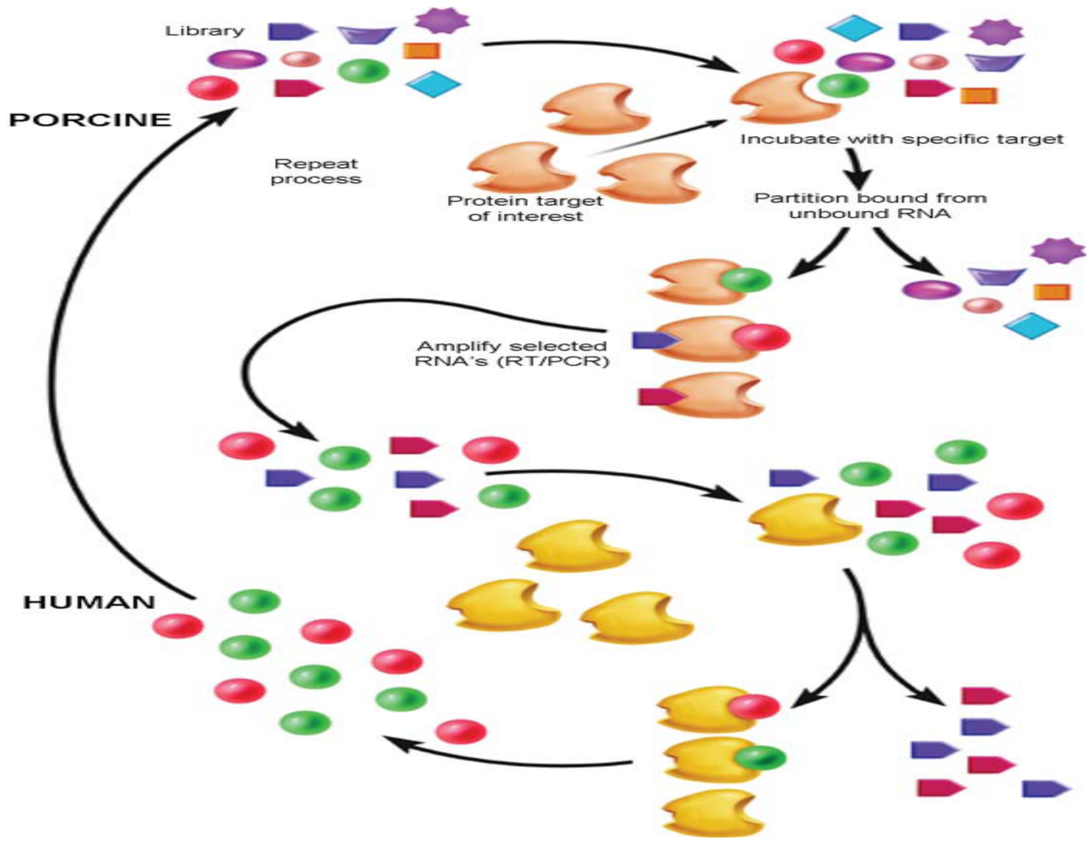


Figure 5. The toggle SELEX procedure (Nimjee et al., 2005).

2.3.5.7 Genomic SELEX

Genomic SELEX is a screening method that uses the whole genome of a certain organism as a screening pool and bioactive molecules as the target. Genomic DNA is fragmented to sufficiently short fragments and the fixed sequences are joined to them. Further selection of sequences binding to the target protein is carried out by the usual methods. The importance of the success of genomic SELEX is the establishment of a good starting pool which should include all the gene sequences and the intron sequences of the genome.

For example, transcription factor Cra was used as the target to gain affinity fragment in *Escherichia coli* genome. Through the analysis of the fragment obtained, ten promoters

were found, six of which were part of genes that could be inhibited by Cra (Shimada et al., 2005).

2.3.5.8 Tailored SELEX.

Tailored SELEX is a screening method that uses a library free of fixed sequences that involve ligation and cleavage of primer site before and after amplification, allowing the isolation of short aptamer sequences that are more readily chemically synthesized and to select aptamers with small size. Tailored selex has been successfully applied to identify aptamers to calcium gene related peptide (Vater et al., 2003; Wen and Gray, 2004).

2.3.5.9 Non SELEX.

Non SELEX is a highly efficient and fast method for aptamers screening. The key point of this method is that it omits the amplification step by PCR. During the different rounds of screening cycles, the random sequences with binding ability separated from the previous cycles are used directly in the next one. The screening process require a higher efficiency of the separation step. Berezovski obtained the high affinity DNA aptamer for h-Ras by only three selection cycles (Figure 6). (Berzovski et al., 2006).

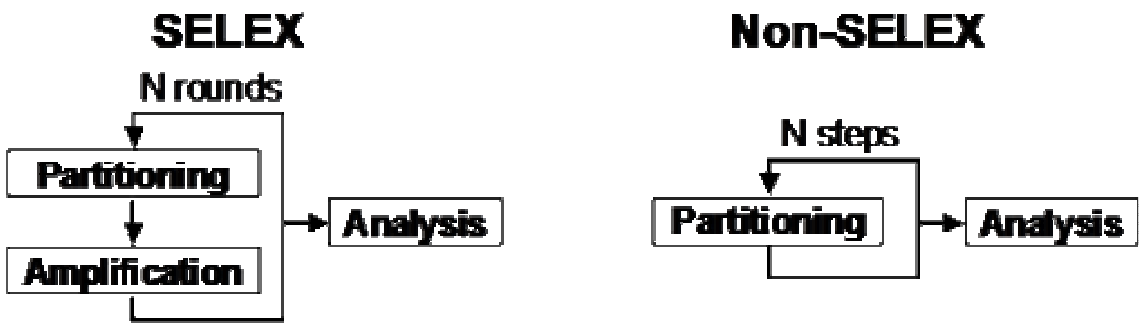


Figure 6. SELEX and non-SELEX procedures (Berezovski, et al., 2006).

This process has a number of advantages over the SELEX procedure. The first advantage is its speed and simplicity: non-SELEX selection takes only about one hour. The second advantage is its ability to accurately determine the abundance of aptamers in the naïve library; this makes it a powerful tool in studies of fundamental properties of DNA libraries. The third and most remarkable advantage of non- SELEX is its potential applicability to nonamplifiable libraries, such as those of DNA- tagged small molecules obtained by DNA- templated synthesis. This feature makes non- SELEX a potentially indispensable tool for drug discovery. For the moment, reports of aptamers obtained by this method are very few (Berezovski, et al., 2006).

2.3.6 Aptamers libraries and complexity.

Starting point of a SELEX process is a chemically synthesized DNA oligonucleotide library that consists of $10^{13} – 10^{15}$ random sequences, comprising a central random region of 30- 60 nucleotides flanked by specific sequences which function as primer binding sites for amplification. The complexity of the library and the maximum diversity equals 4^n, where n is the number of nucleotides in the random sequence. For example, if the random sequence has 35 nucleotide, the complexity is 4^{35} which is around to 10^{21}. The actual degeneracy of a library depends on the amount of DNA used for selection rather than the maximum number of possible sequences (Gold et al., 1995; Famulok and Jenne, 1998).

2.3.7 Aptamers characteristics.

Aptamers have many properties and advantages that make them novel agents for many applications.

2.3.7.1 Control of selection.

The aptamers are in vitro selected, which means that conditions such as pH, ionic strength, temperature and the process of selection are easily controlled. As a result, the properties of aptamers can be changed on demand (Hianik et al., 2006; Musheev and Krylov, 2006).

2.3.7.2 Range of targets.

Aptamers can be developed against a wide range of target molecules. These include whole organisims (Trypanosoma cruzi) (Ulrich et al., 2002), cells (cancer cells and bacterial cells) (Cerchial et al., 2005; Hamula et al., 2008), peptides and proteins (thrombin) (Bock et al., 1992), amino acids (L-arginine) (Geiger et al., 1996), nucleic acids (TAR RNA element of HIV-1) (Boiziau et al., 1999), nucleotides and derivatives (ATP) (Sassonfar and Szostak, 1993), carbohydrates (Cellobiose) (Yang et al., 1998), antibiotics (Tetracycline) (Berens et al., 2001), cofactors (Coenzyme A) (Saran et al., 2003), growth factor (platelet-derived growth factor) (Green et al., 1996), viral proteins (HIV reverse transcriptase) (Tuerk et al., 1992), bacterial spores (anthrax spores) (Bruno and Kiel, 1999), vitamins (B12 vitamin) (Lorsh and Szostak, 1994), and even small inorganic compounds (zinc ion) (Ciesiolka et al., 1995).

2.3.7.3 High affinity.

The high affinities observed for aptamers likely derive from contacts between the aptamer and the target protein over an extended surface of both molecules (Jaeger et al., 1998; Haung, et al., 2003). Aptamers bind to their targets with high affinity comparable to those observed with monoclonal antibodies (Jayasena, 1999). The aptamer-target binding has a dissociation constant (kd) ranging from picomolar to nanomolar

concentrations (Jenison et al., 1998; Watson et al., 2000). For example, the anti-L-selectin aptamer has 60 picomolar affinities, eventhough L-selectin has a negative net charge at physiological pH (Jenison et al., 1998; Watson et al., 2000). The ability of aptamers to form well- folded stable secondary and tertiary structures also contributes to their binding affinity.

2.3.7.4 High specificity.

Aptamers can distinguish between closely related members of a target family, or between different functional or conformational states of the same targets (Sciwert et al., 2000). For example, aptamers to protein kinase C do not interact with closely related isoforms of enzyme that differ by just 4% (Conrad et al., 1994). An aptamer against caffeine has a 10,000 fold lower affinity for theophylline, which differ in just a methyl group (Jenison et al., 1994).

2.3.7.5 Toxicity.

Aptamers exhibit no or little toxicity when administered to the host as therapeutic agents. For example, a single-dose and sub-chronic doses with the aptamer macugen/EyE001 were administered to rats, rabbits, and rhesus monkeys and showed only minimal toxic potency (Drolet et al., 2000; Eyetech study group, 2002, 2003).

2.3.7.6. Immunogenecity

As for immunogenicity, studies on humans and primates showed little or no immunogenic responses. For example, no antigenic response was detected when single and multiple doses of intraviteral injection of anti-VEGF aptamer (macugen/EyE001)

were administered in rhesus monkeys (Eyetech study group, 2002; Vater and Klussmann, 2003).

2.3.7.7 Cost effective

Aptamer production is very cost effective; a single large scale synthesizer can produce up to of 100Kg of oligonucleotide per year. The current cost for aptamer synthesis is estimated at 500 dollars per gram. Continuing improvements in process development are expected to lower the cost to less than 100 dollars per gram in few years (Pendergrast et al., 2005).

2.3.7.8 Modification

One of the major problems effecting the application of aptamers as diagnostic and therapeutic tools is their degradation by nucleases in a relatively short time**.** Chemical modifications of aptamers enhance stability by increasing the resistance of aptamers to nucleases.

Considerable stabilization can be achieved by the addition of capping structures at both the 5' and 3' ends of an oligonucleotide and by the incorporation of modifications to both the ribose and phosphate backbone components (Jellinek, et al., 1995; Pagratis, et al., 1997). Modifications can be engineered into aptamers by either direct incorporation into the random sequence pools or alternatively, by post SELEX experimentation which can include substitution of the 2' hydroxyl group of the ribose backbone of pyrimidines by amino, fluoride or O-methylene groups (Figure.7) (Pagratis et al., 1997; Schmidt et al., 2004).

Modification at the 2' position of the sugar confers nuclease stability, whereas various modifications at the C-5 position of the pyrimidines could be used either to attract certain classes of targets or to generate covalent cross-links with targets (Jayasena, 1999). With

such modifications, the half life of RNA aptamers in biological fluids increases from 8 seconds to 86 hours for fluoro or amino- modified RNA (Ulrich et al., 2004).

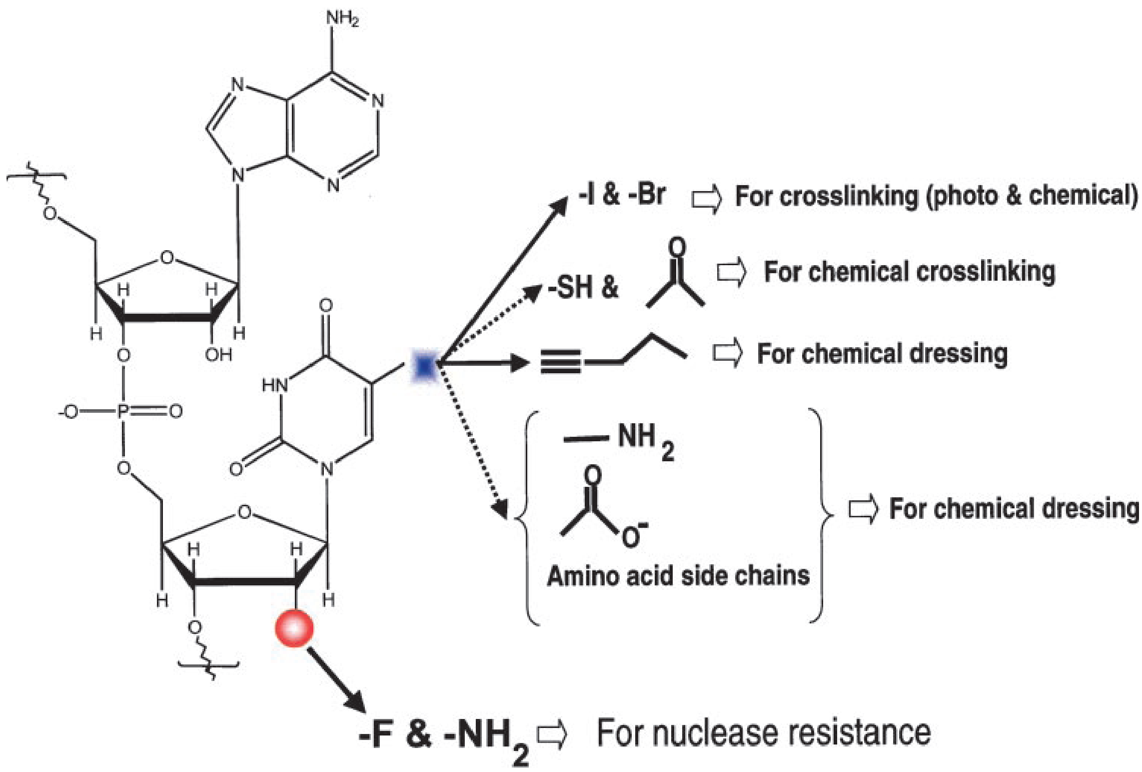


Figure7. Possible modifications (Jayasena, 1999).

2.3.7.9 Antidote availability

An antidote structure is a sequence of nucleic acids that have complementary base-paring to the aptamer. An Antidote- aptamer interaction alters the shape of the aptamer and prevents it from folding into three dimensional conformations necessary for binding to its targets. This aptamer antidote complex is an effective and efficient method of drug control (Figure 8) (Rusconi and Roberts, 2004; Dyke et al., 2006).

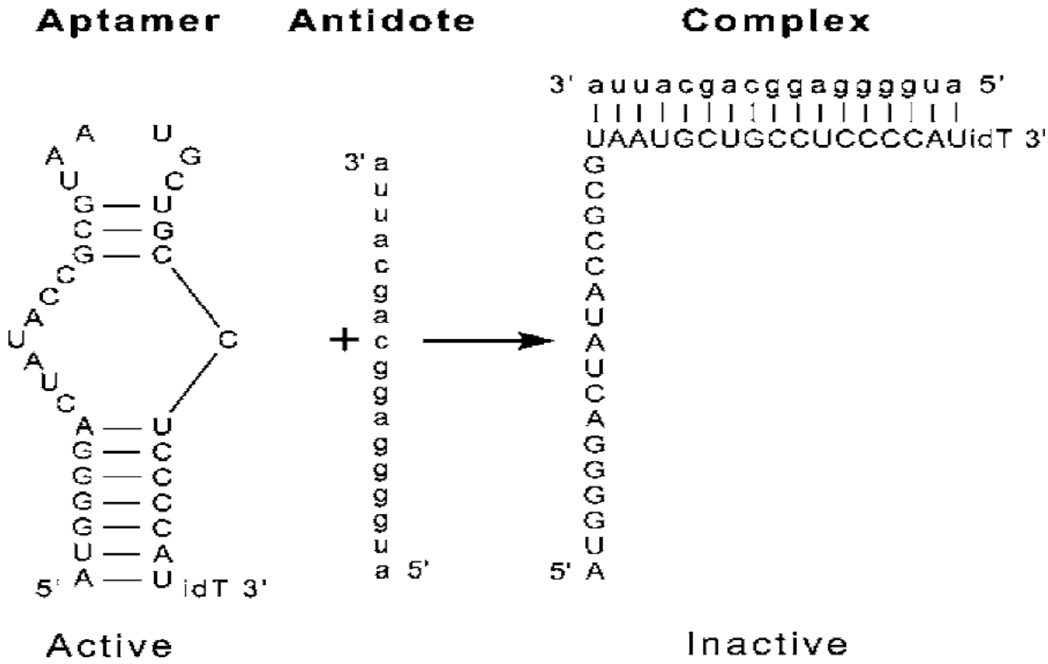


Figure 8. Antidote aptamer interaction (Rusconi and Robrts, 2004).

2.4 Aptamers and the targets

2.4.1 Aptamers to proteins.

Aptamers could modulate the activity of target proteins through their binding to a specific region. The properties of aptamers make them an attractive class of direct protein inhibitors. Their strong and specific binding for a given protein target leads to the potential isolation of ligand to any target and make them an attractive class of molecules that meet and exceed the properties of antibodies (Table 1).

Table 1. The properties of aptamers versus antibodies (Nimjee et al., 2005).

Aptamers	Antibodies
Binding affinity in low nanomolar to picomolar range	Binding affinity in low nanomolar to picomolar range
Entire selection is a chemical process carried out in vitro and can therefore target any protein	Selection requires a biological system, therefore difficult to raise antibodies to toxins (not tolerated by animal) or non-immunogenic targets
Can select for ligands under a variety of conditions for in vitro diagnostics	Limited to physiologic conditions for optimizing antibodies for diagnostics
Iterative rounds against known target limits screening processes	Screening monoclonal antibodies time consuming and expensive
Uniform activity regardless of batch synthesis	Activity of antibodies vary from batch to batch
PK parameters can be changed on demand	Difficult to modify PK parameters
Investigator determines target site of protein	Immune system determines target site of protein
Wide variety of chemical modifications to molecule for diverse functions	Limited modifications of molecule
Return to original conformation after temperature insult	Temperature sensitive and undergo irreversible denaturation
Unlimited shelf-life	Limited shelf-life
No evidence of immunogenicity	Significant immunogenicity
Cross-reactive compounds can be isolated utilizing toggle strategy to facilitate pre-clinical studies	No method for isolating cross-reactive compound
Aptamer-specific antidote can be developed to reverse the inhibitory activity of the drug	No rational method to reverse molecules

2.4.1.1 Aptamers to enzymes.

Thrombin is a key regulatory enzyme in the coagulation cascade. It is a serine protease, produced from prothrombin by the action of factor Xa. A single stranded DNA aptamer prolonged the clotting time from 25 seconds to 169 seconds in the purified protein and from 25 seconds to 43 seconds in human plasma (Griffin et al., 1993; Denanda et al., 1994).

2.4.1.2 Aptamers to cell adhesion molecules.

The selectins, which include L-, E-, and P-selectin, are a family of cell adhesion molecules that are expressed on leukocytes, endothelial cells, and platelets. They have been implicated in a number of inflammatory diseases as well as tissue injury and infection. A 2`-fluropyramidine-modified RNA library was screened by SELEX against P-selectin/IgG (PS-Rg) fusion protein to isolate anti-inflammatory aptamers (Bevilacaua and Nelson, 1993). After twelve rounds of selection, an RNA aptamer with a kd 18 pmol/L did bind to PS-Rg protein on activated human platelets and inhibited both cellular adhesion of PS-Rg to neutrophil and completely blocked neutrophil rolling on activated platelets in vitro (Jenison et al., 1998).

2.4.1.3 Aptamers to growth factors.

Platelet-derived growth factor (PDGF) is a ubiquitous mitogen and chemotactic growth factor. PDGF is involved in wound healing and is linked to the progression of numerous diseases, including atherosclerosis and glomerulonephritis. After twelve rounds of selection, ss DNA aptamers against PDGF were isolated with a Kd value of 50 pmol/L, and inhibited binding of PDGF to its receptors (Green et al., 1996).

2.4.1.4 Aptamers to antibodies and immunological molecules.

Immunoglobulin E (IgE) plays an important role in protecting mammals from parasites. RNA and DNA aptamers were developed against human IgE and yielded aptamers that bind human IgE with high affinities. The aptamers also prevented IgE-mediated cellular degranulation in serum of patients with grass allergy (Weigand et al., 1996).

Complement C5 is a serum glycoprotein that is cleaved to vasoactive C5a and C5b during complement activation and as a central component of inflammation. It subsequently

stimulates neutrophil-endothelial adhesion, cytokine and lipid mediator release and oxidant formation, which are associated with numerous inflammatory states and injury (Morgan, 1995; Mulligan et al., 1997). An RNA library containing 2-fluoropyrimidines was screened by SELEX for those RNA ligands that bind to human C5. Aptamers were identified that bound to human C5 with a Kd of 20–40 nM and inhibited C5 in a human serum hemolytic activity assay (Biesecker et al., 1999).

2.4.2 Aptamers to virus particles

The therapeutic utility of aptamers was first studied in HIV replication. The Tat protein is a potent activator of viral replication. The trans- activation response (TAR) is required for Tat function, that is RNA aptamers for Tat inhibit the HIV replication in vitro.

Hepatitis C virus (HCV) is the main cause of both sporadic and post-transfusion non-A and non-B hepatitis. The viral encoded non structural protein 3 (NS3) which is a serine protease represents a good target for inhibition of HCV. After six rounds of selection, these aptamers bound to NS3 with Kd of 10 nmol/L and inhibited 90% of its helicase and protease activities in vitro (Kumar et al., 1997).

2.4.3 Aptamers to small molecules.

The RNA world hypothesis led to believe the idea that aptamers are capable of binding to small molecules (Maurel and Hanni, 2005). According to this theory, RNA molecules with catalytic properties, so called ribozymes, should also be able to bind cofactors. Moreover, ribozymes might be regulated allosterically by small cellular components. Therefore, aptamers were shown to have the capability to bind small molecules such as ATP (Sassonfar and Szostak, 1993), coenzyme A (Burk et al., 1997), amino acids (Famulok, 1994), carbohydrates (Yang et al., 1998), organic dyes (Ellington and Szostak,

1992), antibiotics (Wallis and Schroeder, 1997), toxins (Hirao et al., 1997), or ions (Ciesiolka et al., 1995).

2.4.4 Aptamers to cells and whole organisms.

The protozoan Trypanosoma cruzi is responsible for Chaga's disease, which primarily affects the nervous system and heart and is often fatal if untreated. RNA aptamers were developed which bind to parasite receptors for host cell matrix molecules on the trypomastigote cell surface. The SELEX technique can be performed with living cells and it can be verified without knowledge of all elements of a target's surface. Specific ligands can be selected and subsequently analyzed (Ulrich et al., 2002).

SELEX provides the opportunity to select aptamers that recognize unique molecular signatures of cancer cells by cell-SELEX. In 2006, Shangguan et al. isolated DNA aptamers that bind to T cell acute lymphoblastic leukemia (ALL) cell lines. Ramos cell line was used as a negative control cell line to reduce the collection of DNA sequences that could bind to common surface molecules present on both types of cells (Shangguan et al., 2006; Phillips et al., 2008).

2.5 Applications of aptamers.

During the past 20 years, aptamers have been developed for various applications such as analytical, diagnostics, drug development, target validation and therapeutics (Zhou and Rossi, 2010).

2.5.1 Analytical applications of aptamers.

2.5.1.1 Chromatography.

Aptamers hold a great promise as molecular recognition tools for their incorporation into analytical devices and, in particular, they can be used as immobilised ligands in separation technologies (Clark and Remcho, 2002).

Aptamers specific for the thyroid transcription factor 1 (TTF1) have been tested in several assays comprising affinity chromatography and it has been recently recognized as a highly specific marker for primary lung adenocarcinomas. Selected aptamers against TTF1 are modified with biotin and attached to streptavidin magnetic beads. The possibility of protein purification of the native form of a protein without relying on affinity tags that could affect the protein structure and function was presented as one of the major advantages for the aptamer-based affinity chromatography (Murphy et al., 2003).

2.5.1.2 Mass spectrometry.

Aptamers as substrates for protein capture and analysis in affinity matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) have been recently described. A DNA thrombin-binding aptamer has been used for direct detection of thrombin, after covalent binding to a fused-silica glass surface. A comparison with a scrambled oligonucleotide that cannot form the characteristic G-quartet demonstrated that thrombin

is captured only by the specific aptamer. Different amounts of thrombin, from 5 to 50 pmol, were incubated with the aptamer spots. The results showed optimal detection at 10 pmol of thrombin with a detectability varying among aptamers spots, but reaching 5 pmol in the best cases. The cycle of capture, release and analysis could be repeated on the same spot by simply unfolding the aptamer upon application of low pH matrix. The aptamer-modified surface was able to selectively capture thrombin also from a complex matrix such as human plasma (Dick and McGown, 2004).

2.5.1.3 Signal aptamers.

Molecular beacons are oligonucleotide probes that can detectt the presence of specific nucleic acids and they are widely used in real time PCR. Signaling aptamer is the attachment of a fluorophore at a specific position of an aptamer which can undergo a conformational change induced by the binding of the target (Jhaveri et al., 2000).

One of the first aptamer beacons was reported by Yamamoto and Kumar (2000), who developed an aptamer specific for Tat protein of HIV-1. A duplex RNA aptamer was split into two oligomers, one transformed into a DNA probe and the other, partly complementary to the DNA probe, was designed to form a hairpin structure that contain fluorophore (fluorescein) and quencher (4(4_-dimethylamino-phenylazo)benzoic acid) at both ends. In the absence of Tat, the two nucleic acids molecules exist independently, and in the presence of protein, the beacon changes its structure to interact with the other half of the aptamer for binding to Tat. The opening of the hairpin structure causes physical separation of the fluorophores–quencher pair, resulting in a fluorescence enhancement (Figure 9) (Yamamoto and Kumar 2000).

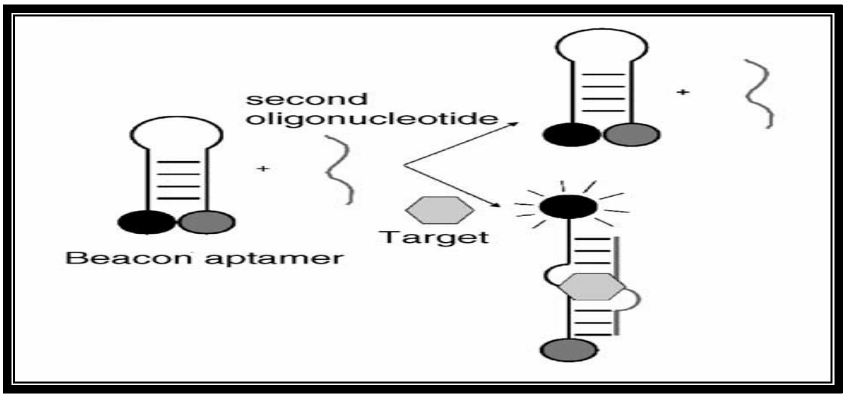


Figure 9. Signaling aptamer for Tat protein (Tombelli et al., 2004).

2.5.1.4 Capillary electrophoresis.

The separation and detection advantages of aptamers make them a very attractive affinity probe in capillary electrophoresis- based quantitative assays of proteins. Electrophoresis can be used to efficiently separate free aptamers from the aptamer–target complex due to the changes in electrophoretic properties which can arise from structural transitions or changes in size when the aptamer interacts with the target molecule (German et al., 1998; Kotia et al., 2000).

Capillary electrophoresis (CE) has been also presented as an alternative separation method directly during the SELEX process (Mendonsa and Bowser, 2004). An aptamer specific for IgE (Wiegand et al., 1996) has been selected using CE as a powerful separation technique to discriminate between bound and free DNA. Different clones were obtained after two or four selection rounds with Kd ranging from 27 to 84nM which are only modestly higher than what is obtained with conventional SELEX (*K*d of 6 nM) after 15 rounds of selection. After only two rounds of CE-SELEX, around 100% of sequences in the DNA pool was bound IgE. This rate of enrichment is very high if compared to the one obtained with conventional SELEX (Cox et al., 2002).

2.5.1.5 Biosensors.

The application of aptamers as biocomponents in biosensors offers, over classical affinity sensing methods mainly based on antibodies, a multitude of advantages, such as the possibility to easily regenerate the function of immobilized aptamers. Several electrochemical methods for protein detection have made use of aptamers. The aptamers are usually coated on nanoparticles (NPs) or a gold-electrode surface with streptavidin before exposing the functionalized surface to a sample containing the target (Hamula et al., 2006).

Thrombin is the most commonly studied protein in using electrochemical methods. Xiao et al., (2005) developed "signal on" and "signal off" formats for detecting thrombin using immobilized aptamers on an electrode for binding and using methylene blue (MB) for electron transfer.

For the signal-on format, a rigid duplex DNA structure prevents the methylene blue (MB)-tagged oligomer from reaching the surface of the electrode. Thrombin binding destabilizes the duplex DNA structure by forcing the aptamer into the characteristic G-quartet structure, resulting in electron transfer from MB on the liberated oligomer to the electrode (Figure 10).

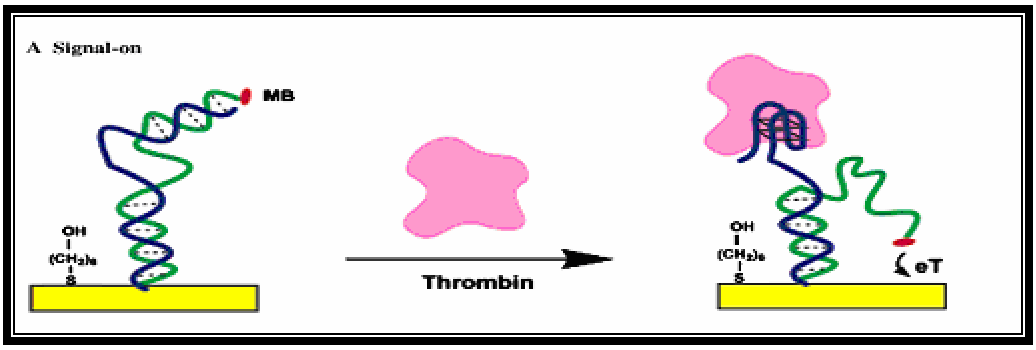


Figure 10. Signal on for thrombin in electrochemical sensors (Hamula et al., 2006).

For the signal-off architecture, the MB-tagged aptamer is initially able to reach the electrode surface and generate electrical signal. Upon thrombin binding, a conformational

change in the aptamer results in the removal of the MB from the electrode surface and inhibition of the electron transfer (Figure 11).

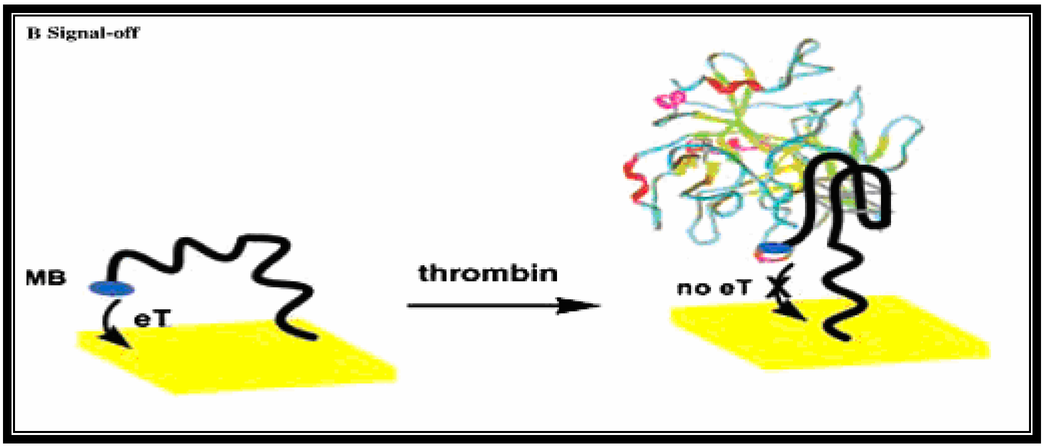


Figure 11. Signal off for thrombin in electrochemical sensors (Hamula et al., 2006).

Stojanovic and Landry (2002) used an aptamer specific for cocaine to develop a colorimetric probe for this molecule. This simple assay could result in the development of simple and inexpensive colorimetric analytical methods. A pool of 35 different dyes was first screened to examine their binding to the aptamer in the presence or absence of cocaine. One of these dyes, diethylthiotricarbocyanine iodide, was chosen to construct the colorimetric sensor. After incubation of the dye with the aptamer, cocaine addition causes a displacement of the dye with an attenuation of absorbance proportional to the concentration of the added cocaine (Figure 12). (Stojanovic and Landry, 2002).

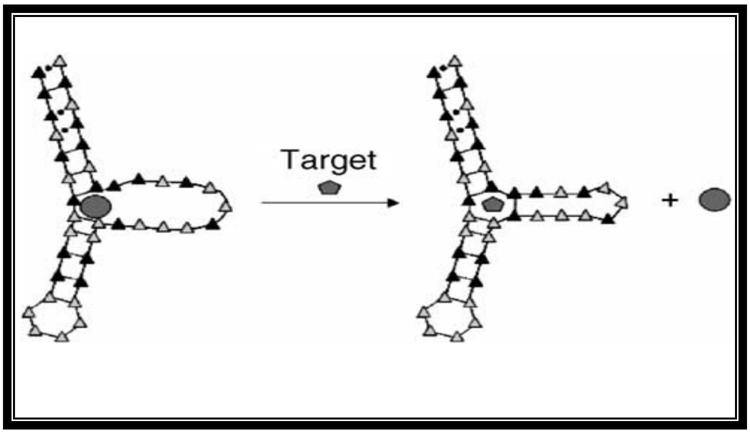


Figure 12. Colorimetric probe for detection of cocaine (Stojanovic and Landry, 2002).

An aptamer based assay has been recently developed with a ‘‘dipstick’’ format where detection results can be seen by the naked eye and do not need sophisticated instruments. Liu et al., 2006 developed such aptamer-based dipstick assay for cocaine (Liu et al., 2006).

2.5.2 Therapeutic applications of aptamers.

Aptamers can interact tightly and specifically with their targets. This interaction may lead to different effects on the function of target molecules, which could allow aptamers to be readily used as pharmaceutical agents. The year 2004 was a milestone year for therapeutic applications of aptamers, when the united states Food and Drug Administration FDA, approved pegaptanib sodium "Macugen" , which is a specifc anti-vascular epithelial growth factor (anti-VEGF 165) RNA aptamer with a dissociation of constant is 50 picomolar that used for treatment age related macular degeneration (AMD). In phase I clinical trials, , 0.25- 3.0 mg injection of Macugen were used in 15 patients with exudative AMD that did not reach dose limiting toxicity, and 80% of these patients had improved vision or stabilized vision after three months. A phase II study involving AMD patients undergoing repeated injections of Macugen revealed that 88% of patients had stabilized or improved vision after three months (Gragoudas et al., 2004; Eugene et al., 2004).

Obviously, the successful results of this new drug could promote the investigation toward introduction of more new therapeutic aptamers (Pendergrast et al., 2005; Christopher and Kelland, 2006).

2.5.3 Aptamers as delivery vehicles for other molecules.

Aptamers targeting cells provide the means to develop drug delivery vehicles that specifically address certain malignant subtypes. In this regard; aptamers have been applied as guiding modules in multifunctional complexes to target distinct cell types and tissues. The prostate specific membrane antigen (PSMA) is a cell surface molecule associated with the onset and progression of prostate cancer. Modification of anti-PSMA aptamers with additional molecules allowing their selective aptamer- triggered delivery to cancer cells, polymer coated nanoparticles with encapsulated chemotherapeutics have been attached to the 5'- amino end of the aptamer and the aptamer specifically localized the complex in a solid tumor where the adjacent chemotherapy induced a reduction in the size of the tumor up to total remission (Farokhzad et al., 2004; Farokhzad et al., 2006).

Beside chemotherapeutics, small interference RNA molecules (siRNA) can also be used to assemble siRNA- aptamer conjugates, thereby facilitating the cell- specific delivery of siRNA. Recently, Rossi and co-workers used aptamer-based siRNA – delivering agents where they fused anti-gp120 binding aptamers with siRNA that are specific for the HIV transcript tat and rev. Treatment of HIV-1 infected cells with these conjugates resulted in the specific down regulation of the appropriate mRNA transcript (Cheng et al., 2007; Mayer, 2009).

2.5.4 Diagnostic applications of aptamers.

Given the high affinity and specificity for the target molecules, aptamers could have potential application in a variety of detection and diagnostic systems. For example, fluorescence labeled aptamers can be used to detect and measure serum amino acids such as phenylalanine, which is useful in diagnosis of hyperphenylalaninemia (Osborne et al., 1997). Another method that is applicable to aptamer based detection is enzyme-linked

oligonucleotide assay (ELONA). This technique is basically an enzyme linked immuneosorbent assay (ELISA), in which aptamers are used instead of antibodies to detect the target molecules. Drolet et al (1996) developed ELONA to quantify human vascular endothelial growth factor (hVEGF) levels in serum by using an aptamer specific to hVEGF as the affinity ligand in a typical sandwich ELISA. A monoclonal anti- VEGF antibody was used as the microtiter plate-bound capture reagent, and a fluorescein-tagged aptamer bound to human VEGF served as the detection reagent. The capture antibody was first immobilized onto microtiter plates and left overnight. Samples were then added and allowed to complex with the immobilized antibody. Unbound products were then removed by washing, and a fluorescein-labelled aptamer (rather than a second antibody which is used in ELISA) was allowed to bind to the human VEGF; thus completing the "sandwich". The human VEGF165 was then quantitated by measuring the amount of labelled aptamer bound to the matrix through the use of a chemiluminescence detection system. The precision, specificity, and accuracy of the method were studied and were well within the limits expected from a typical enzyme-linked assay (Drolet et al., 1996).

2.5.5 Aptamers that regulate gene expression.

Aptamers can be used in regulation of gene expression and in gene therapy. For example HIV-1 has 57 nucleotide long hairpin (trans activation response,TAR) element which is located at the very 5` end of the retroviral mRNA and serves as the binding site for the HIV protein Tat and for two cellular proteins, cyclin T1 and Cycline dependent kinase 9 (CDK-9) (karn et al, 1999; Rana and Jeang, 1999). This Tat-associated kinase phosphorylates the C-terminal domain of RNA polymerase and consequently trans-activates the transcription of the viral genome. DNA aptamers selected against the HIV-1 TAR RNA element form RNA/DNA kissing complex with TAR loop that compete for

the binding of proteins with TAR loop and consequently inhibit the transcription of the virus (boiziau et al, 1999; collin et al, 2000).

2.6 Kinases

The eukaryotic protein kinases family is one of the largest protein families represented in the human genome that control many biological processes involved in cell growth and differentiation, gene expression, membrane transport and apoptosis (Till and HubBard, 2004; Schijen and Giriffin, 2002). It constitutes 2% of the whole eukaryotic genome (Huse and Kuriyan, 2002).

Kinases are enzyme that transfer phosphate group from adenosine triphosphate (ATP) to determined target proteins. (Singer et al., 2004). The human genome is believed to contain 518 putative protein kinase genes; of which 430 genes are expressed to be catalytically active. The vast majority of kinases are serine/ threonine kinases and tyrosine kinases (Jianying and Xun, 2003; Ortutay et al., 2005).

2.6.1 Tyrosine kinases

Tyrosine kinases are enzymes that catalyze the transfer of the γ- phosphate of ATP to the hydroxyl group of tyrosine residues on protein substrates (Kostich et al., 2002; Paul et al., 2004). Tyrosine kinases play an important role in cell division, growth and death (Singer et al., 2004). One of the normal functions of tyrosine kinases is the regulation of hematopoisis and hematopoietic stem cell function. Subsequently, many tyrosine kinases may become oncogenic as a result of genetic alterations that induce constitutive kinase activity leading to cell transformation and cancer (Huse and Kuriyan, 2002).

Tyrosine kinases are primarily classified as receptor tyrosine kinases (RTK) such as PDGFR, FLT-3, and FGFR, and non receptor tyrosine kinases (NRTK) such as ABL and

JAK-2 (Paul et al., 2004). Receptor tyrosine Kinases are transmembrane glycoproteins that play an important role in regulating intracellular signaling pathways, and are responsible for the development of many cancers. Mutated tyrosine kinases have an important role in inducing many malignances, especially hematological malignances. Examples include BCR-ABL fusion transcripts seen in the vast majority of chronic mylocetic leukemia (CML) patients, as well as the constitutive activation of other oncogenes such as platelets derived growth factor receptor (PDGFR) in chronic myelomonocytic leukemia patients, FLT3 in acute mylocetic leukemia (AML) patients, c-Kit in systemic mastocytosis and gastrointestinal stromal tumers, and Janus Kinase 2 in chronic myeloproliferative disorders (Paul et al., 2004; Chalandon et al., 2005; Campbell and Green., 2006; Gilliand and Levine, 2007).

A cascade of signals is initiated from receptor tyrosine kinases after ligand binding to extracellular domains, which lead to receptor dimerization, kinase activation and autophosphorylation of tyrosine residues (Schijen and Griffin, 2002).

Class III receptor tyrosine kinases share sequence homology and a similar overall structure with five immunoglobulin- like repeats in the extracellular domain, a single transmembrane domain (TM), a juxtamembrane domain (JM), two intracellular tyrosine kinase domains (TK1 and TK2) divided by a kinase insert domain (KI), and a C-terminal domain (Figure 13) Mutations to this group of receptors have been linked recently to the pathogenesis of myeloid malignancies (Reilly, 2002).

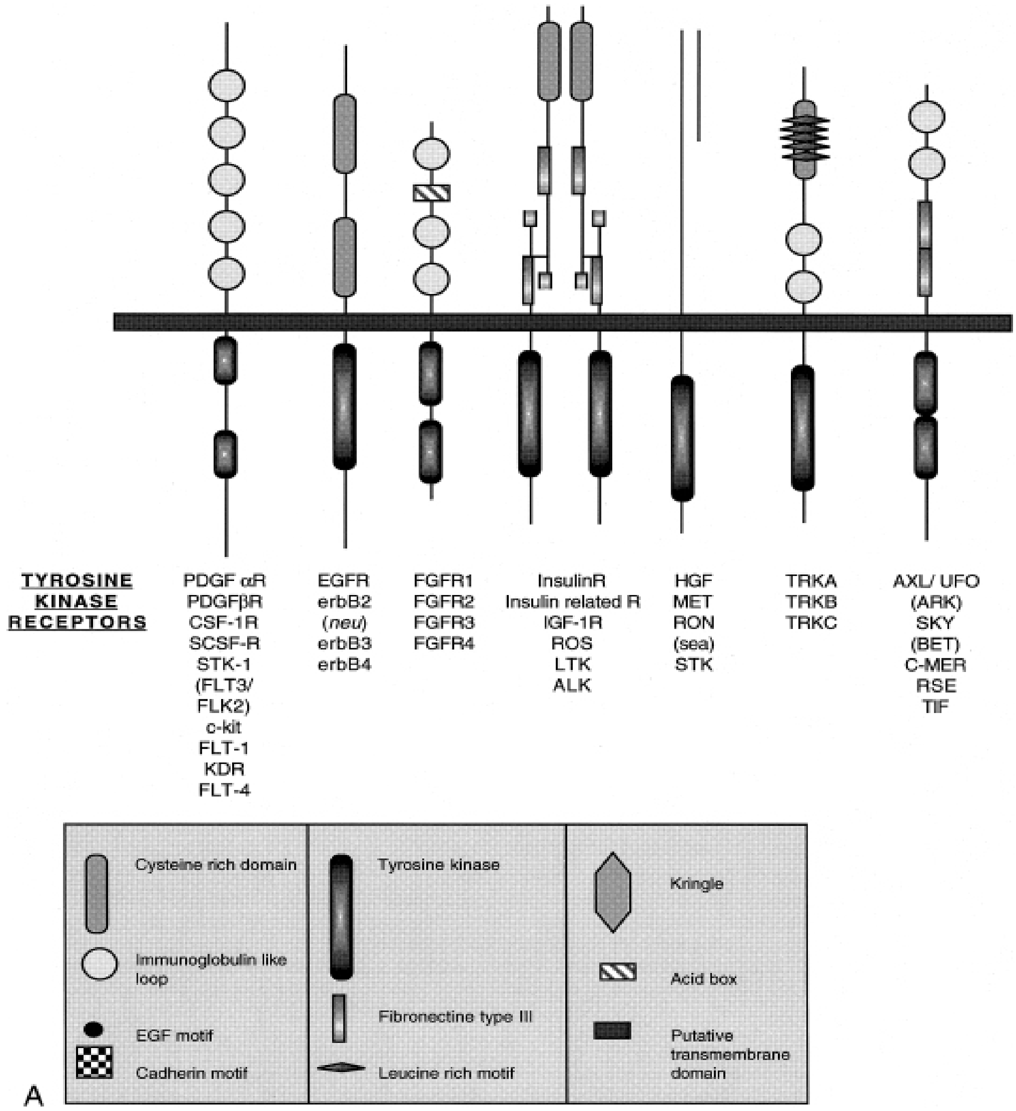


Figure 13. Structure of tyrosine kinase receptors (Yarden and Ullrich, 1988).

2.6.2 Platelet- Derived Growth Factor Receptor

Platelet derived growth factor receptor (PDGFR) is a member of a family of protein kinases that is encoded by two genes (*PDGFRA and PDGFRB*). The *PDGFRA* gene is located on chromosome 4q12 and has a molecular size of 170 KDa, where as the *PDGFRB* gene is located on chromosome 5q33 with a molecular size of 180 KDa. The PDGFR consists of two distinct polypeptide subunits; α and β (Henrik and Westermark, 1999; Uehara et al., 2003).

PDGFR is a type III receptor tyrosine kinase, and is characterized by the presence of

three main parts; an extracellullar ligand binding domains, which consists of five immunoglobulin like domains, single transmembrane hydrophobic domain and the intracellular cytoplasmic kinase domain (Pietras et al., 2002; Tamborini et al., 2006).

PDGFR α and PDGFRβ are two highly related receptor tyrosine kinases showing approximately 75% homolgy between the two intracellular kinase domain. PDGFRs are expressed on erythroid and myeloid precursors in bone marrow, monocytes, megakaryocytes, endothelial cells, osteoblast and glial cells (Relliy, 2003; Swick et al., 2008).

2.6.2.1 Platelet-derived growth factor receptors dimerization.

Activation of PDGFRs require binding of platelet derived growth factor (PDGF) as a ligand to the receptor and autophosphorylation of tyrosine kinase domain. PDGF is composed of three biologically active protein forms: AA, BB and AB forms. PDGF molecules bind two receptor molecules simultaneously, since the α- receptor binds both A and B chains where as β- receptor binds only the B chain with high affinity. The different isoforms of PDGF have different combinations of receptor dimers. Thus, PDGF-AA induces αα receptor dimers, PDGF-AB induces αα or αβ receptor dimmers and PDGF- BB induces all three possible combination of receptor dimers (Figure 14) (Heldin et al., 1998; Victor et al., 2001; Tallquist and Andrius, 2004).

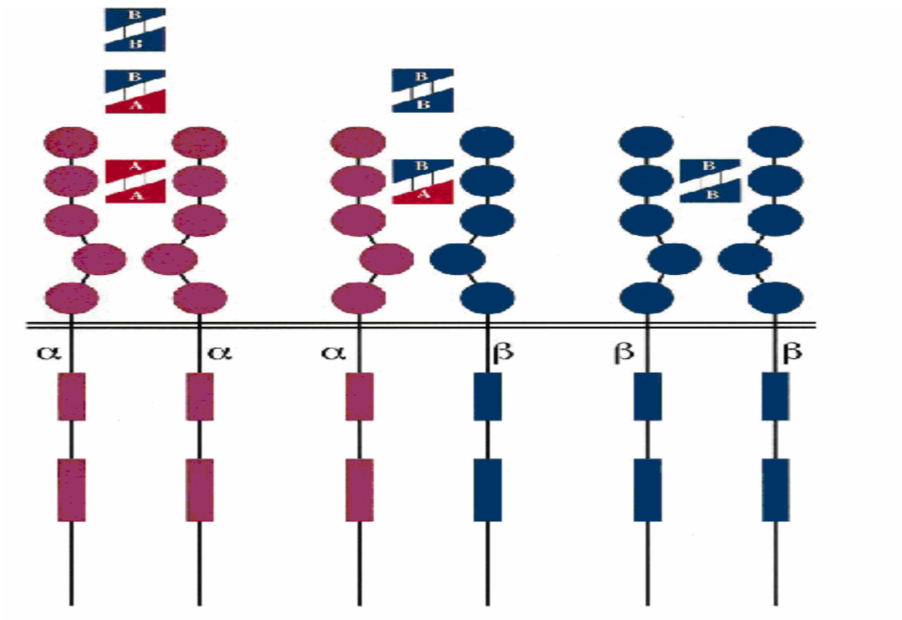


Figure 14. Ligand induced dimerization of PDGF receptors (Heldin et al., 1998).

2.6.2.2 Platelet-derived growth factor receptors activation and signal transduction pathways:

Ligand binding induces receptor dimerization and autophosphorylation. An important function of autophosphorylation is to regulate the catalytic activity of the kinase. The autophosphorylation site of the α receptor on the tyrosine 849 and in β receptor on the tyrosine 857, which allows binding and activation of cytoplasmic Src homology 2 (SH2) domains containing signal transduction molecules, such as phosphatidyl inositol 3 kinase (PI3K) and phospholipase Cγ (PLCγ). Thereby, a number of different signaling pathways are initiated leading to cell growth, actin reorganization, migration, angiogenesis and differentiation (Funa et al., 2003; Puglianiello et al., 2004; Huang et al., 2007).

Activation of PDGFRs has also been shown to inhibit some apoptotic pathways in normal and tumor cells and can lead to malignant transformation; hence inhibition of PDGFR activation may decrease cell proliferation and increase the rate of cell death (Uehara et al., 2003; Swick et al., 2008).

2.6.2.3 Genetic abnormalities of PDGFR

Tyrosine kinase activation of PDGFR plays an important role in regulation of cell growth. So abnormalities in these proteins might lead to constitutive activation of PDGFR and may have many biological mutations.

Expression of both receptors is increased in atherosclerotic blood vessels and in many human carcinomas including those of malignant glioma, lung cancer, prostate cancer and ovarian tumors (Funa et al., 2003). Chromosomal translocations in the PDGFRs have been implicated in hematological malignancies (Figure 11), such as fusion of FIP1L1/ PDGFRα which is present in 50% of hypereosinophilic syndrome (HES) patients, and fusion of TEL/ PDGFRβ which is present in chronic myelomonocytic leukemia (CMML) patients (Carroll et al., 1997; Funa et al., 2003; Elizabeth et al., 2005).

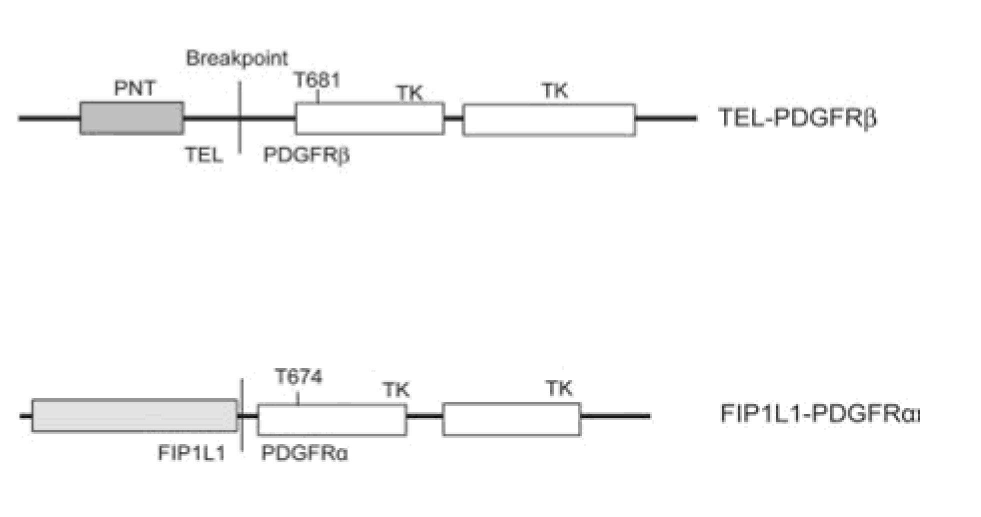


Figure 15. Genetic abnormalities in PDGFRβ and PDGFRα (Elizabeth et al., 2005).

2.7 Aims of the study:

This study aims to:

- Develop high affinity aptamer ligands that bind to PDGFRα and PDGFRβ.
- Establish of theoretical and practical experiences in SELEX technology in our laboratory.
- Analyse the aptamer sequences, and determine of the conserved sequences that can play an important role in the inhibitory effect of the selected aptamer.

3. Materials and methods.

3.1 Instruments and equipment:

- Thermal cycler (MJ Research, USA).
- Gel documentation system (UVP, USA).
- Spectrophotometer (Smart Spec TM plus, BIORAD, USA).
- Electrophoresis chambers (Clever Scientific, UK).
- Electrophoresis power supply (Power Pac Basic TM, BIORAD, USA).
- Ultra-viol transilluminator (Vilber Lourmat, France).
- Autoclave (Selecta, Spain).
- Incubator (flii Galli, Italy).
- Centrifuge (Biofuge, Heraeus, Germany).
- Digital balance (Denver, UK).
- Vortex mixer (HARRIS, UK).
- Pipettes (Witopet, Germany).
- PCR tubes (Bio basic inc, Canada).
- Magnetic separator (Promega, USA).

3.2. Reagents.

- Active PDGFR α kinase domain (poly His-tagged) (millipore, USA).
- Active PDGFR β kinase domain (poly His-tagged) (millipore, USA).
- Magne His Protein Purification system (Promega, USA).
- ssDNA library (Medland certified company, USA).
- Synthesis of aptamers (Medland certified company, USA).
- Primers (Medland company, USA).
- Taq DNA polyemerase (Bio basic inc, Canada).
- PCR buffer 10X (Bio basic inc, Canada).
- Deoxy nucleiotides triphosphate (dNTPs) (Promega, USA).
- Nuclease free water (Promega, USA).
- DNA ladder (Promega, USA).
- PGEM-T and PGEM-T easy vector cloning system (Promega, USA).
- *E-coli* strain MJ107 (Fermantas, France).
- LE-agarose powder (Promega, USA).
- Low melting point agarose (Promega, USA).
- TE-saturated phenol (Promega, USA).
- Plasmid extraction kit (Bio basic inc, Canada).
- DNA plasmid transformation kit (Promega, USA).
- Ligase enzyme (Fermantas, France).
- Ligase buffer 2X (Fermantas, France).
- TE buffer (Promega, USA).
- Tris-Borate-EDTA 10X (Bio Basic Inc, Canada).
- Loading dye (Promega, USA).
- Ethidium bromide (Promega, USA).

- Absolute ethanol (Gainland Chemical Company, UK).
- NaCl (Promega, USA).
- Sodium acetate (Reidel-de Haen, Germany).
- LB media (agar) (Invitrogen. USA).
- LB media (broth) (Invitrogen. USA).
- Ampicillin powder (Promega, USA).
- 5-Bromo-4-chloro-3-indolyl-β-D galactoside (Xgal) (Bio Basic Inc, Canada).
- Isopropyl-1-thio-β-D-Galactoside (IPTG) (Bio Basic Inc, Canada).
- $MgCl_2$ (Promega, USA).
- EDTA (Invitrogen. USA).
- Di-Methyl Sulphoxide (DMSO) (Gibco, USA).
- QIA quick PCR purification kit (QIAGEN, USA)

3.3. Methods:

3.3.1. ssDNA library and primer design:

The initial template was obtained via chemical synthesis using cyanoethyl phosphoroamitide chemistry by Medland Company, USA

DNA fragments were 90 bases in length, with 45 nucleotides random region flanked with defined sequences of the forward and reverse primers, and restriction sites for Hind III and EcoRI restriction enzymes.

5`GGGATGGATCC AAGCTT ACTGG **45N** GGG AAGCTT CGATAG GAATTC GG 3'

Hind III Hind III EcoRI

Where N is one of four deoxynucleotides; A, T, C or G. The library was designed to give theoretically 4^{45} different sequences, which represents about $1.24x10^{27}$ different sequences.

The sequences of the forward and reverse primers were as follows in Table 2:

Table 2. Sequences of forward and reverse primer.

primer	Direction	Sequence
APT-F	forward	5' -GGGATGGATCCAAGCTTACTGG -3'
APT-R	reverse	5'- CCGAATTCCTATCGAAGCTTCCC -3'

3.3.2. Small scale amplification:

In order to provide multiple copies of the original template and to eliminate damaged ones, the chemically synthesized oligonucleiotide pool is first amplified enzymitically by Polymerase Chain Reaction (PCR) before initiating a selection experiment. Optimal amplification conditions can be determined before large-scale amplification to prepare the initial pool of dsDNA by carrying out small-scale trials.

The optimal conditions were as follows:

One hundred nanograms of the initial template were used in the small scale amplification step which represents about $21x10^{13}$ different sequences in 50 µL of total volume PCR reaction in PCR mix containing PCR buffer, 200 µM of each dNTPs mix (Promega, USA), 1 µM of the forward primer 5' GGGATGGATCCAAGCTTACTGG 3' and 1 µM of the reverse primer 5'-CCGAATTCCTATCGAAGCTTCCC-3' (Medland company, USA), 2.5 mM $MgCl_2$, 2.5 unit of Taq DNA polymerase (Promega, USA), and nuclease free water up to 50 µL. The PCR program was as follows: heating up to 95°C for three minutes as an initial denaturation step, then thermally cycling seven times for 15 second at 95°C, 15 seconds at 50°C (annealing temperature), 20 second at 72°C (extension step). These seven cycles were then followed by a final extension step at 72°C for two minutes.

3.3.3. Large scale amplification:

Ten micrograms of the chemically synthesized template were amplified to prepare the initial pool of the double stranded DNA (dsDNA), which represents approximately 2 x 10^{16} different sequences, in 5000 µL of total volume. The PCR reaction mix was divided into 100 PCR tubes, each containing 50 µL of PCR reaction mix with same conditions optimized in small-scale PCR as described above.

3.3.4. Small scale asymmetric PCR:

To produce ssDNA from dsDNA we use asymmetric PCR method which is a method that uses different primer ratios; to produce one strand more than the other when amplified by PCR.

The small-scale amplification by asymmetric PCR was used to optimize the preparation of initial pool of ssDNA. It was performed by amplifying 5 µL of dsDNA PCR product obtained from the large-scale amplification in 50 µL of total volume PCR reaction in

PCR mix containing PCR buffer, 1 μM of the forward primer, 0.02 μM of the reverse primer, 200 μM of each dNTPs mix (Promega, USA), 2.5 mM $MgCl_2$, 1.5 unit of Taq DNA polymerase (Promega,USA).Fore asymmetric PCR, the reverse to forward primers ratio used is 1: 50, which gave good yield of ssDNA. The PCR program was as follows: 3 minutes at 95^{o}C for initial denaturation, then 25 cycles of 15 seconds at 94^{o}C, 15 seconds at 55^{o}C, 20 seconds at 72^{o}C, followed by a final extension step of 2 minutes at 72^{o}C.

3.3.5 Large scale asymmetric PCR:

Twenty percent of the double stranded oligonucleotide pool were amplified by asymmetric PCR in 5000 μL of total volume PCR reaction mix divided into 100 PCR tubes each containing 50 μL total volume in the same PCR conditions described in small-scale asymmetric PCR.

3.3.6. Agarose gel electrophoresis:

PCR products from all amplification reactions were detected by electrophoresis in 4% agarose gel.

3.3.6.1. Preparation of 4% agarose gel

An adequate volume of electrophoresis 1X TBE buffer was prepared by dilution of one volume of 10X TBE buffer (Promega, USA) in nine volumes of distilled water. Then, 2 grams of agarose powder were added (Promega,USA) into 50 mL of 1X TBE buffer and the mix was melted in a microwave with swirling to ensure even mixing and clear appearance. Then, 10 μL of 2.5mg/ml ethidium bromide was added to the mix and poured into the mini gel casting platform and allowed to harden.

3.3.6.2. Loading the samples:

The gel casting platform containing the set gel was placed in the electrophoresis tank soaked in sufficient volume of 1X TBE buffer. About 10 μL of the PCR product were loaded into the well. Each run has 3 μL of DNA ladder mixed with 3 μL of loading dye (Promega, USA) to determine the size of the bands of the PCR product. To begin the electrophoresis, the leads were connected with the power supply and the gel was run at 110 volt for twenty minutes. The DNA was visualized by placing on a UV light source and it was photographed directly.

3.3.6.3Preparation of 4% low melting point agarose gel

Two grams of low melting point agarose (Promega, USA) were melted in 50 mL 1X TBE buffer by following the same way as the 4% agarose gel was prepared in.

3.3.7. Extraction of ssDNA fragments from low melting point agarose gel:

After the asymmetric PCR reaction was run, the ssDNA and dsDNA were produced. The ssDNA yield must be extracted efficiently in order to produce pure single stranded oligonucleotide library used in SELEX rounds. The samples were loaded into 4% low melting point agarose gel and electrophoresed, and the method of extracting the ssDNA yield was as follows:

The desired bands of ssDNA were cut out using sterile scalpel and placed in 1.5 mL microcentrifuge tubes, then 300 μL of 1X TBE buffer (Promega, USA) were added and the tubes were heated at 65^{o}C for 30-45 minutes until the gel dissolved. An equal volume of TE saturated phenol (pH 8.2) was added to the tubes and mixed on a vortex for about 1

minute in order to produce a milky appearance solution; and then centrifuged for 15 minutes at 13,000 rpm.

After centrifugation, the supernatant was taken to a new microcentrifuge tube and 2.5 volumes of absolute ethanol and 0.1 volume of 3M sodium acetate were added, and the mixture was incubated at -20 ^{o}C overnight to enhance the precipitation of ssDNA. On the next day the mixture was centrifuged for 15 minutes at 13,000 rpm. Then, the supernatant was discarded and the small transparent pellet was washed with 700 µl of 70% ethanol to remove residual phenol and salts, followed by centrifugation for 10 minutes at 13,000 rpm. Then, the pellets were rehydrated with binding buffer. This pool is ready to be used in the iterative cycles in the SELEX procedure.

3.3.8 In vitro selection method:

3.3.8.1. Folding of ssDNA library.

The ssDNA library was heated at 95ºC for 10 minutes in 200 µl binding buffer which contains 100mM HEPES (pH 7.5), 10 mM imadazole, 1 mM $MgCl_2$ and 150 mM NaCl (Promega, USA). Then, the mix was rapidly cooled to 4ºC for 5 minutes and then incubated on ice for 15 minutes to insure proper folding of ssDNA ligands into its three dimensional structures, after which it was equilibrated at room temperature for about 10 minutes.

3.3.8.2. Negative selection:

The folded ssDNA ligands were incubated with 15 µl of magne His Ni-particles magnetic beads (Promega, USA) on 37ºC for 30 minutes, then ssDNA that bind to the anti-HIS magnetic beads were separated by a magnetic field separator and the supernatant was collected. The negative selection process was performed in the first five rounds of

selection in order to pick up any sequence that would bind to beads before being bound to the target of interest and to eliminate false background selection of high affinity ligands to Ni-particles only.

3.3.8.3. Incubation of active enzymes with HIS tagged PDGFR kinase domain:

After the negative selection processes were performed, the HIS tagged kinase domain of both enzymes PDGFRα and PDGFRβ (2μg of each enzyme for the first round, 1μg for the next rounds and 0.5μg for the last round) were incubated with 15μL of magnetic beads for 15 minutes at 37°C to insure appropriate binding between the HIS tagged PDGFR kinase domain and the Ni particles magnetic beads. After that, the supernatant which contained the ssDNA was added to the mixture and incubated for 90 minute at 37°C.

PDGFRα and PDGFRβ were run together at similar concentrations in the same reaction tube up to the first five rounds of selection in order, to elicit, if present, similar domains that the aptamer will bind to.

After binding, the ssDNA-PDGFR-Ni were washed three times with 200 μL of binding buffer for each washing for the first five rounds, and six times for the next five rounds to remove any non specific binders and to enhance efficient partition.

3.3.8.4. Elution of ssDNA:

Separation of PDGFR-ssDNA complex from Ni-PDGFR-ssDNA complex was performed by addition of 30 μL of elution buffer containing 100 mM HEPES (pH 7.5), 500 mM imidazole, and 0.5 M NaCl (Promega, USA) and incubation for 10 minute at 90°C. Then

the ssDNA that bind to the target was separated by a magnetic field separator and the supernatant was collected and amplified.

3.3.8.5. Amplification of ssDNA eluate

Because of the high complexity of the initial oligonucleotide library, it is normally expected to get only few functional oligonucleotides as a result of the selection step. In order to provide multiple copies of the eluted ssDNA; ssDNA was amplified by PCR using the same conditions described above except for the number of cycles which was 15 cycles instead of 7, and then performing asymmetric PCR in the same conditions described above to produce ssDNA candidate mixture for the next round of selection.

3.3.8.6. Negative control PCR.

After each round, a negative control of PCR was performed to ensure that there is no contamination that interferes with the enrichment process of aptamers which gives false selected sequences later in cloning and sequencing.

3.3.8.7. Rounds of selection

Ten rounds of SELEX were performed to select anti-PDGFRα/ anti PDGFRβ aptamers with conditions summarized in Table 3

Table 3 Rounds of SELEX condition

Round	Number of PCR cycles	Quantity of ssDNA µg	Quantity of PDGFRα/β at the same run µg	Quantity of PDGFRα µg	Quantity of PDGFRβ µg	Incubation temperature	Number of washing	Incubation time minutes
1	15	-	4 (2 for each enzyme)	_	_	37°C	3	90
2	15	2	2 (1 for each enzyme)	_	_	37°C	3	90
3	15	2	2 (1 for each enzyme)	_	_	37°C	3	90
4	15	2	2 (1 for each enzyme)	_	_	37°C	3	90
5	15	2	2 (1 for each enzyme)	_	_	37°C	3	90
6	15	1	_	1	1	37°C	6	90
7	15	1	_	1	1	37°C	6	90
8	15	1	_	1	1	37°C	6	90
9	15	1	_	1	1	37°C	6	90
10	15	1	_	0.5	0.5	37°C	6	90

3.3.9 Purification of PCR product:

After the tenth round of selection, the obtained aptemer should be purified from other components of the final dsDNA product. The purification was performed following the QIAquick PCR purification kit (QIAGEN, USA) as follows:

Five volumes of the binding buffer were added to 60 µL of PCR product and mixed, and then the sample was applied to the QIAquick column and placed in a provided 2 ml collection tube and then centrifuged for 30 seconds at 13,000 rpm. The flow-through was discarded and the columns were washed with 750 µL of binding buffer and then centrifuged for 1 minute at 13,000 rpm. Again, the flow-through was discarded and the columns were centrifuged for another minute. In order to elute the purified DNA, 50 µl of

elution buffer were added to the QIAquick columns placed on clean 1.5 microcentrifuge tubes and centrifuged for 1 minute at 13,000 rpm. After that, the eluted DNA was collected and stored at -20^{o}C.

3.3.10. Cloning.

By iteratively completing the procedures of selection, amplification and conditioning during the SELEX process, the complexity of the original library is reduced and target-binding candidates are enriched. Therefore, the final pool of the ten selection rounds was cloned into the bacterial vector PGEM-T to determine the sequence of each individual aptamer, and the process was as follows:

3.3.10.1. X-Gal preparation.

Preparation of 3% X-Gal was performed by dissolving 0.1gram of X-Gal powder (Bio Basic Inc, Canada) in 3 mL of DMSO (Gibco, USA) to a final concentration of 33 mg/ml.

3.3.9.2. IPTG preparation.

Preparation of IPTG was performed by dissolving 1gram of IPTG powder (Bio Basic Inc, Canada) in 50 mL of autoclaved distilled water to a final concentration of 10 mM.

3.3.10.3. LB solid media preparation.

Preparation of LB solid media was performed by dissolving 16 grams of LB powder in 500 ml of distilled water followed by heating the media until completely dissolved. Then, the media was autoclaved for 30 min at 121°C and after cooling the media to 50°C; about 25 mg of ampicillin powder (50 µg/ml) , 5 ml of 10 mM IPTG (0.1 mM) and 0.3 ml of X-Gal were dissolved in the media. Then, after well mixing, the media was poured into Petri dishes and stored at 4°C.

3.3.10.4. LB broth media preparation.

Preparation of LB broth media was performed by dissolving 2 grams of LB broth powder in 100 mL of distilled water followed by heating the media until complete dissolving of powder. The media was then autoclaved for 30 min at 121°C. After cooling the media to 50°C, 5 mg of ampicillin powder was added to a final concentration of 50µg/ml and then poured into sterile 1.5 mL microcentrifuge tubes (1ml/tube) and stored at 4°C.

3.3.10.5. Preparation of *E.coli* competent cells.

Under aseptic conditions; a loop full of *E. coli* was incubated in 0.5 mL of liquid LB media without ampicillin. An inoculating loop was used for streaking the mixture on nutrient agar plate which is then incubated overnight at 37°C. Then, one colony of bacterial culture was moved from overnight nutrient plate using inoculating loop into 1 mL of transformaide C-Medium (Fermantas, France). The culture was suspended by gently mixing and incubation of the tube in a shaker at 37°C for 2 hours.

3.3.10.6. Ligation

Ligation of the purified dsDNA to the PGEM-T vector was performed following the protocol of the PEGM-T and PEGM-T easy vector system kit (Promega, USA), by mixing 3 µL of the PCR product with 1 µl of T4 DNA ligase enzyme and 1 µl of PEGM-T and PEGM-T easy vector plus 5 µL of 2X Rapid Ligation buffer. The mixture was incubated 1 hour at room temperature.

3.3.10.7. Transformation and growth

After preparation of E-coli competent cells from a fresh culture plate in C-Medium as described above, the tube was spun at 13, 000 rpm for 1 minute at room temperature, then the supernatant was discarded and the pelleted cells were resuspend in 300 µL of T-solution and incubated on ice for 5 minutes. The cells were spundown again for 1 minute at 13,000 rpm at room temperature and then the supernatant was removed and the cells were resuspended in 120 µL of T-solution and then incubated on ice for 5 minutes. During that, LB-ampicillin agar plates are pre-warmed at 37°C for 20 minutes.

Preparations of DNA for transformation was performed by taking 5 µl of ligation mixture into new microcetrifuge tube and placing it on ice for 2 minutes. Then 50 µl of the resuspended cells were added to the tube that contains DNA and incubated on ice for 5 minutes. Then, the cells were plated on pre-warmed LB-ampicillin agar plates and incubated overnight at 37°C. After growing the transformed cells, white and blue colonies were screened to determine the successful cloning; the white colonies indicate successful cloning.

3.3.10.8. Preparation of plasmid for sequencing

Plasmid extraction was performed by the Pure Yield^{TM} Plasmid miniprep System (promega, USA) done by incubation of each white colony in 1 ml of LB-ampicillin broth media overnight at 37°C on shaking platform.

Six hundred microliters of the bacterial cells grown in LB medium were lysed by the addition of 100 µL of cell lysis buffer, and the tubes were mixed by inversion. Then, 350 µL of neutralization solution were added and the tubes were centrifuged for 3 minutes at 13,000 rpm. After that, the supernatant of each tube was transferred to a PureYield TM minicolumn and placed into a PureYield TM collection tube, and then the collection tubes were centrifuged for 15 seconds at maximum speed. After that, the flow through

was discarded and 200 μL of Endotoxin Removal Wash were added and centrifuged at maximum speed for 15 seconds. This step was preceded by the addition of 400 μL of Column wash solution to the microcolumns and centrifuged again at 13,000 rpm for 30 seconds. The microcolumns were transferred to clean 1.5 mL microcentrifuge tubes and 30 μL of Elution Buffer was added to each tube, let to stand at room temperature for 1 minute and then centrifuged for 15 seconds at maximum speed. The eluate containing the purified plasmid DNA was collected and stored at -20°C.

3.3.11. Sequencing

Extracted plasmids were sent for sequencing with the forward universal sequencing primer M13. The sequencing reactions were performed by Macrogen Inc., South Korea. After sequencing, twenty four aptamer sequences were obtained from twenty four successful sequencing reactions.

3.3.12. Synthesis of aptamer sequences

Each aptamer sequence was obtained via chemical synthesis using cyanoethyl phosphoroamitide chemistry (Medland certified company, USA) in 50 nanomole scale.

3.3.13. Predicting the secondary structure of aptamers.

For the prediction of the secondary structure of single stranded nucleic acids the mfold software developed by Michael Zuker which is available at the web site http://www.bioinfo.rpi.edu/applications/mfold (Zuker, 2003)was used. This software takes into consideration the folding conditions used in the selection process which includes 1 mM $MgCl_2$, 150 mM NaCl, and 37°C as incubation temperature.

4. Results.

4.1 Results of small and large scale dsDNA amplification.

The chemically synthesized oligonucleotides were amplified enzymitically by PCR in order to provide multiple copies of the original templates and to eliminate damaged templates. Optimal amplification conditions were determined by small-scale PCR amplification prior to large-scale PCR amplification. The result of amplification was a 90bp long fragment as seen in Figure 16.

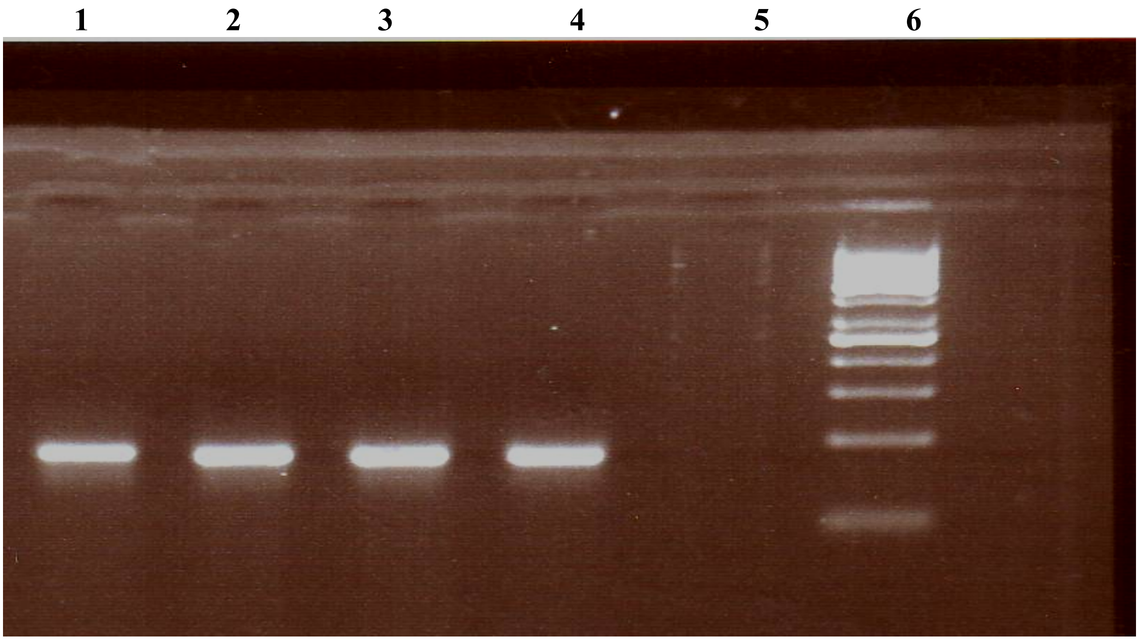


Figure 16. 90bp PCR product of small scale (lane 1 and 2) and large scale (lane 3 and 4) amplification. Lane 5 represents negative control, guided by 50bp ladder (lane 6).

4.2 Results of asymmetric PCR amplification.

Synthesis of the first ssDNA library was performed by trial extension reactions to optimize the optimal conditions and forward to reverse primers ratio. These trials showed that the best ratio of forward to reverse primers is 50:1 as seen in Figure 17.

1 2 3 4 5 6

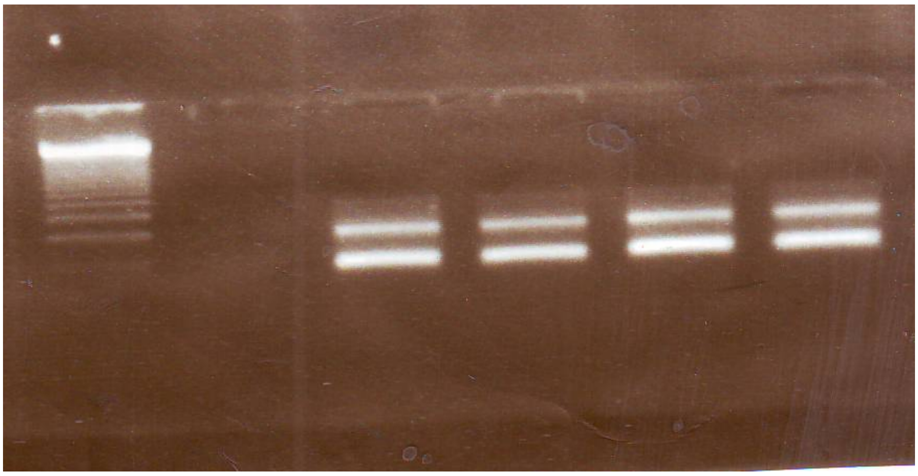


Figure 17. asymmetric PCR product of small scale (lane 3 and 4) and large scale (lane 5 and 6) amplification. Lane 2 represents negative control, guided by 50bp ladder (lane 1).

4.3 PCR products of first five rounds of selection.

Ten rounds of SELEX processes were performed to select anti-PDGFRα and anti-PDGFRβ. In the first five rounds of selection the PDGFRα and PDGFRβ were run in the same round to elicit, if present similar domains that the aptamer will bind to. About 10 µl of PCR product of each round were loaded into 4% of agarose gel and the results are shown in Figure 18.

7 6 5 4 3 2 1

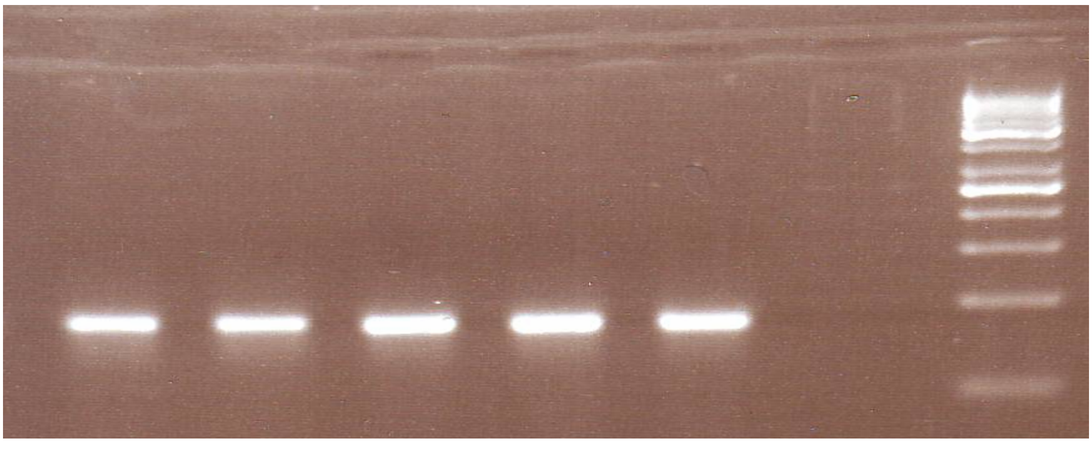


Figure 18. PCR product of the first five rounds of selection respectively (from lane 3 to 7). Lane 2 represents negative control and lane 1 represents the ladder.

4.3.1 PCR products of next five rounds of selection.

After round number 5; it was noted that there is an increase in the yield of the PCR product, which indicates an increase in the ssDNA eluted after incubation with the target. So, the PDGFRα and PDGFRβ were run separately to provide specific aptamer sequences.

PCR product of each round are shown in Figure 19.

1 2 3 4 5 6 7 8 9 10 11

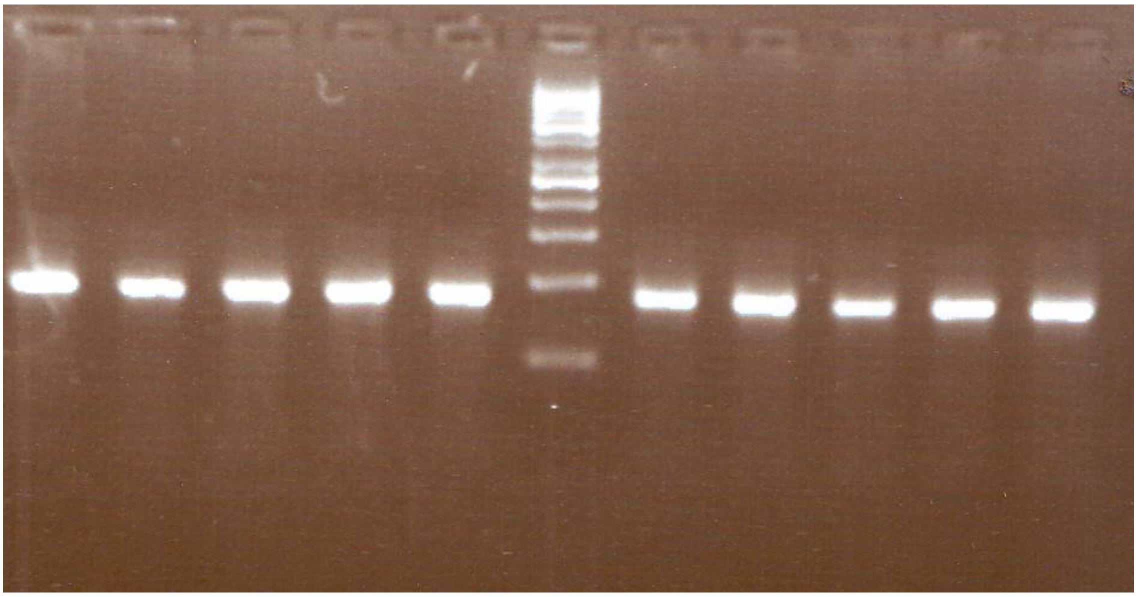


Figure 19. PCR product after second five rounds of selection (lanes 1 to 5 represent sixth to tenth round against PDGFRα while lane 7 to 11 represent sixth to tenth round against PDGFRβ , guided by 50bp ladder.

4.3.2 PCR products of ninth rounds of selection.

In the ninth round of selection, different amounts of PDGFRα and PDGFRβ (1 μg and 3μg) were used with a fixed amount of ssDNA (1 μg) in the selection precesses without any differences in the selection process conditions. After amplification of the eluted ssDNA, the ssDNA that was exposed to the higher protein quantity gave a stronger band compare to one exposed to the less quantity. This may indicate increase the binding of the ssDNA during the selection procedure when increasing the amount of the target (Figure 20).

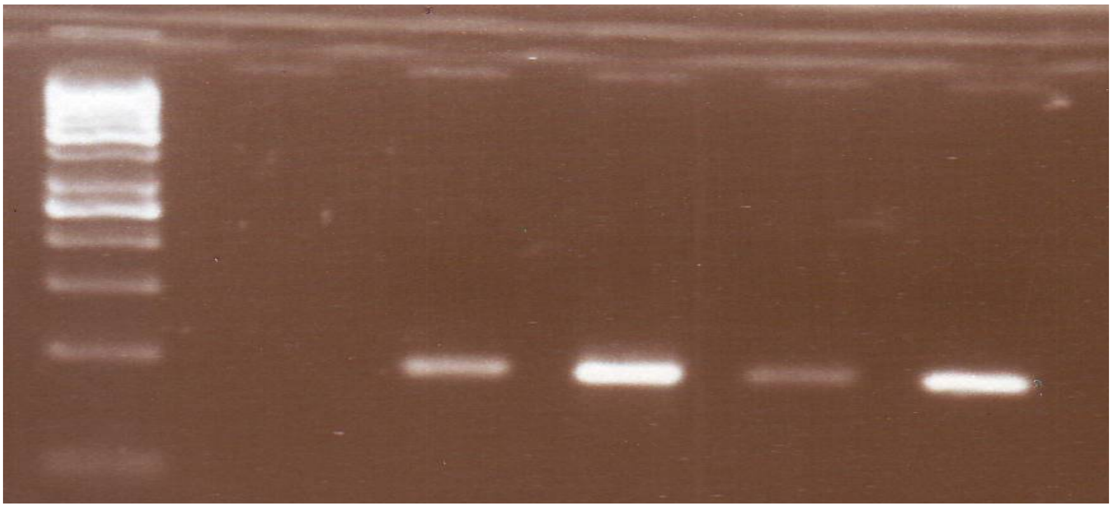


Figure 20. PCR amplification for the ninth round of selection. Lane 3 represent 1 µg of anti PDGFRα aptamers. Lane 4 represent 3µg of anti PDGFRα. While lane 5 represent 1 µg of anti PDGFRβ aptamers. Lane 4 represent 3µg of anti PDGFRβ. Lane 2 is the negative control and guided by 50bp ladder.

4.4 Sequencing results.

About 24 different sequences of aptamers were obtained from sequencing of 40 successful cloned plasmids; 10 for PDGFRα (Table 4), and 14 for PDGFRβ (Table 5).The sequencing results were highly accurate and monitored by precise quality control system (Figure 21).

Table 4. Full sequences of PDGFRα selected aptamers

Name of sequence	Sequences from 5' to 3'
PDGFR-α 1	GGGATGGATCCAAGCTTACTGG**TGGTGCGTGTGTGGGAGGGAGGTGGGGGGTAGGATGGGCTCCT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-α 2	GGGATGGATCCAAGCTTACTGG**TAGGTCTCGGCGGTGGTGGAGGGATGGGATGGGATGGGGCGAGGT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-α 3	GGGATGGATCCAAGCTTACTGG**CAACACTAAGTACATGCGGGCGTTGCGGCGGGGGAGGGGAGGGTA**GGGAAGCTTCGATAGGAATTCGG
PDGFR-α 4	GGGATGGATCCAAGCTTACTGGTGGT**GGCGAGCGGGTGGGCTGGAGGGTGGAGGTCTTGGAGGGCGT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-α 5	GGGATGGATCCAAGCTTACTGGT**GGTGGCGACCCGGCGGGCGGGAGGGTGGAGGTTGGACGCCCT** GGGAAGCTTCGATAGGAATTCGG
PDGFR-α 6	GGGATGGATCCAAGCTTACTGG**GGGGGGTGGATCGCGGGAGGGCGGGTCAACATGGAGTAGACACTT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-α 7	GGGATGGATCCAAGCTTACTGG**CAACACTAAGTACATGCGGGCGTTGCGGCGGGGGAGGGGAGGGTA**GGGAAGCTTCGATAGGAATTCGG
PDGFR-α 8	GGGATGGATCCAAGCTTACTGG**AGGACAGACGTGGTATACACTGGTGGTGCCTAGACGTTCGCGTG**GGGAAGCTTCGATAGGAATTCGG
PDGFR-α 9	GGGATGGATCCAAGCTTACTGG**GGGGTGATCAGTATGCGTTGGTCGTGATGGCGCGGGGGCCGG**GGGAAGCTTCGATAGGAATTCGG
PDGFR-α 10	GGGATGGATCCAAGCTTACTGG**TGGTGCGTGTGTGGGGGGGAGGGAAGGGTAATGCGAGTGTCCTA**GGGAAGCTTCGATAGGAATTCGG

Table 5. Full sequences of PDGFRβ selected aptamers

Name of sequence	Sequences from 5' to 3'
PDGFR-β 1	GGGATGGATCCAAGCTTACTGG**TGGTGGCGAGCGGGTGGGCTGGAGGGTGGAGGCTTGGAGGGCGT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 2	GGGATGGATCCAAGCTTACTGG**GCTATGGCGGTGGTGAGTGGGAGGTGGGATGGGGGACGGACGATG**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 3	GGGATGGATCCAAGCTTACTGG**CACATGGGGTGTCAGGCGATACCAGCAACGGTAGGAATGTGTATA**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 4	GGGATGGATCCAAGCTTACTGGT**CAAATGGTGTTGGTGGTTGGGGGATATGGGCGGTGGGAGGTACT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 5	GGGATGGATCCAAGCTTACTGG**GCTATGGCGGTGGTGAGTGGGAGGTGGGATGGGGGATGGACGATG**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 6	GGGATGGATCCAAGCTTACTGG**TGGCGGCGCGTGGGAGGGAGGTGGGGGGTAGGATGGGCTCCTA**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 7	GGGATGGATCCAAGCTTACTGG**CGCAAGTGTAAGTTCAGCGGTGATGCGGGTGGCCGTAAATATCGT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 8	GGGATGGATCCAAGCTTACTGG**TGTGCGGTGAGAGGGAGGAGGGCGGGCGGCGGTCACTGGTCGAAT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 9	GGGATGGATCCAAGCTTACTGG**TGGTGGCGAGCGGGTGGGCTGGAGGGTGGAGGTCTTGGAGGGCGT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 10	GGGATGGATCCAAGCTTACTGG**GCTATGGCGGTGGTGAGTGGGAGGTGGGATGGGGGATGGACGATG**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 11	GGGATGGATCCAAGCTTACTGG**TGTGGCGGCGTGTGGGAGGGAGGTGGGGGGTAGGATGGGCTCCTA**GGGAAGCTTCGATAGGAATTCGG

PDGFR-β 12	GGGATGGATCCAAGCTTACTGG**TGGTGGCGAGCGGGTGGGCTGGAGGGTGGAGGTCTTGGAGGGCGT**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 13	GGGATGGATCCAAGCTTACTGG**GTGAGGAGGGAGGGGGAGGAGGTGGTCGCATGTGGTTGTGGTGAC**GGGAAGCTTCGATAGGAATTCGG
PDGFR-β 14	GGGATGGATCCAAGCTTACTGG**CGGTGAGTGGTGGGATGGGAGGGAGGGATGGGAGCCTGACTTGTG**GGGAAGCTTCGATAGGAATTCGG

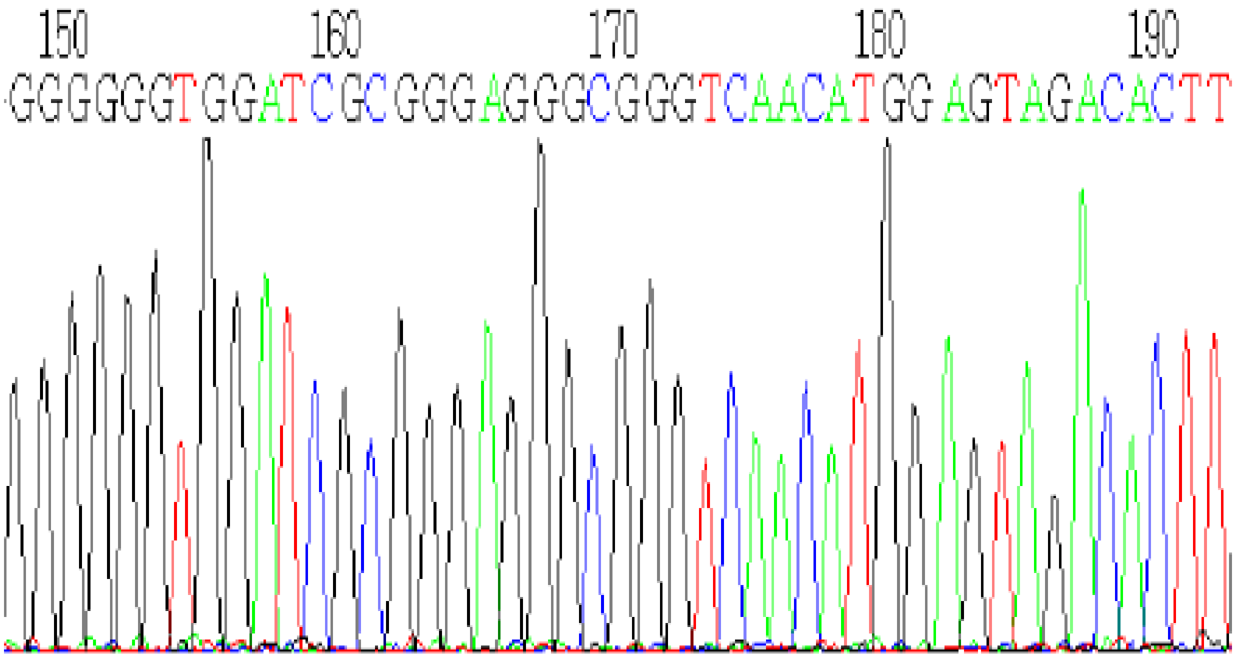


Figure 21. Electropherogram of random region of PDGFRα6

After cloning and sequencing of the successfully cloned plasmid, the selected sequences of anti-PDGFRα/β aptamers were obtained. Tables (6 and 7) describe the molecular weight of anti-PDGFR α/β aptamers, and the percentage of different nitrogen bases in the selected sequences. The tables clearly show that the most frequent base is the guanine (G).

Table 6. Anti- PDGFR α aptamers informative data.

Name of sequences	Molecular weight (kilo Dalton)	Frequency of nucleotide			
		A%	C%	G%	T%
PDGFRα1	28.96	17	12.5	47.7	22.8
PDGFRα2	29.663	18.9	12.2	47.8	21.1
PDGFRα3	29.562	23.3	16.7	42.2	17.8
PDGFRα4	29.655	16.7	13.3	48.9	21.1
PDGFRα5	28.884	17.1	19.3	45.5	18.2
PDGFRα6	29.577	22.2	15.6	43.3	18.9
PDGFRα7	29.562	23.3	16.7	42.2	17.8
PDGFRα8	29.027	22.5	18	37.1	22.4
PDGFRα9	28.534	17.3	16.1	44.9	21.7
PDGFRα10	29.259	20.2	12.3	45	22.5

Table 7. Anti- PDGFR β aptamers informative data.

Name of sequences	Molecular weight (kilo Dalton)	Frequency of nucleotide			
		A%	C%	G%	T%
PDGFRβ1	29.349	16.9	13.5	49.5	20.1
PDGFRβ2	29.688	20	12.2	49.5	20.1
PDGFRβ3	29.391	26.7	16.6	35.6	21.1
PDGFRβ4	29.523	20	11.1	43.4	25.5
PDGFRβ5	29.687	20	11.1	47.8	21.1
PDGFRβ6	28.987	18.2	14.8	47.8	21.1
PDGFRβ7	29.357	23.3	16.7	36.7	23.3
PDGFRβ8	29.609	20	15.6	45.6	18.8
PDGFRβ9	29.655	16.7	13.3	48.9	21.1
PDGFRβ10	29.687	20	11.1	47.8	21.1
PDGFRβ11	29.639	17.8	13.3	47.8	21.1
PDGFRβ12	29.655	16.7	13.3	48.9	21.1
PDGFRβ13	29.687	20	11.1	47.8	21.1
PDGFRβ14	29.647	20	12.2	46.7	21.1

4.5 Primary and secondary structures of selected aptamers.

The secondary structures of anti-PDGFR aptamers were predicted by computer modeling using the Mfold program which takes in consideration the factors that affect the folding of nucleic acid sequences and the conditions of incubation, such as temperature of incubation and ionic strength (Zuker, 2003). Each sequence showed different free energy (dG) which reflects the stability of folding structure of aptamer. The small free energy means the folded structure is more stable. Figures from 22 to 45 show the most stable secondary structure for each selected aptamer.

Figure 22. The most favorable secondary structure for anti- PDGFRα1 aptamer

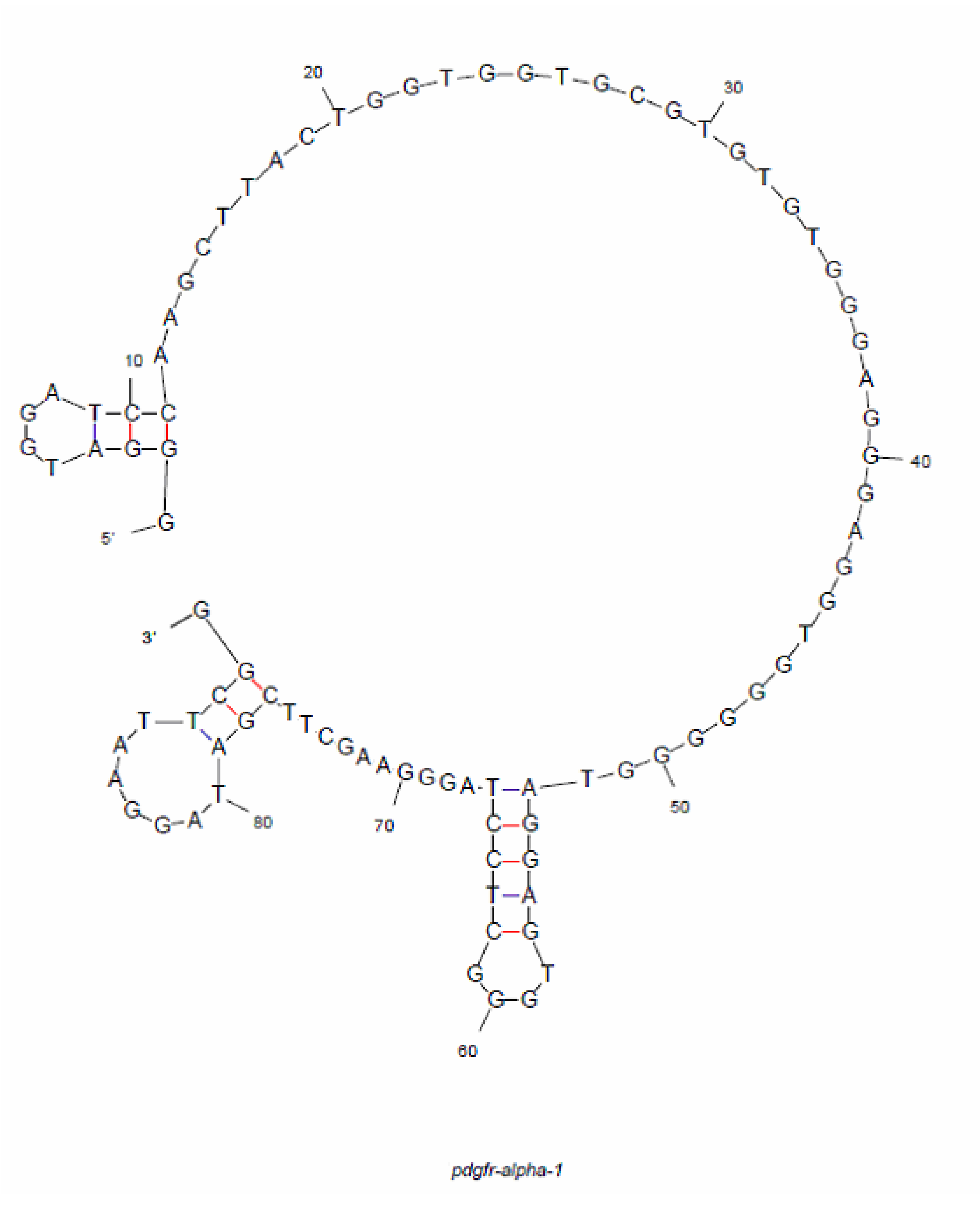


Figure 23. The most favorable secondary structure for anti- PDGFRα2 aptamer

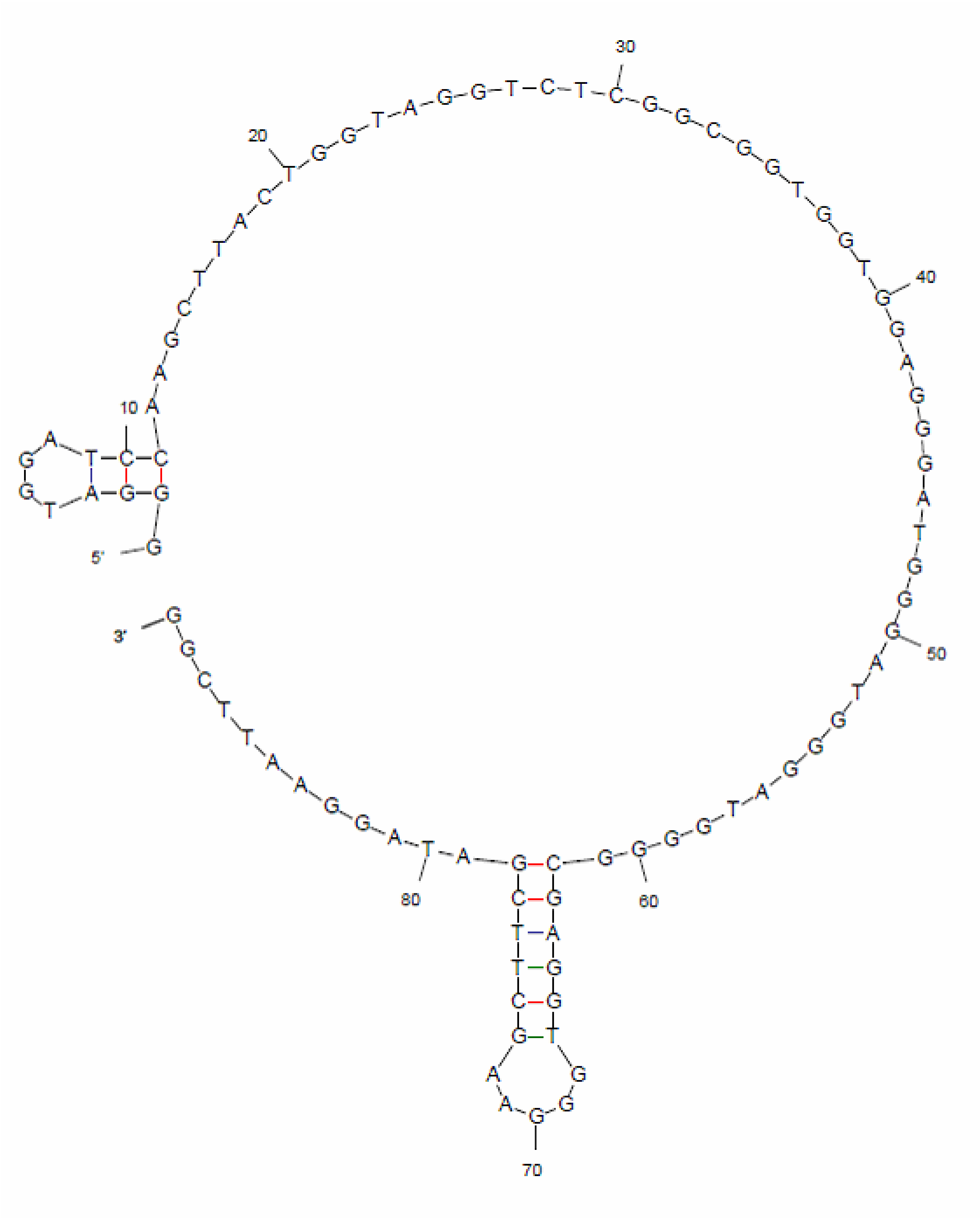


Figure 24. The most favorable secondary structure for anti- PDGFRα3 aptamer

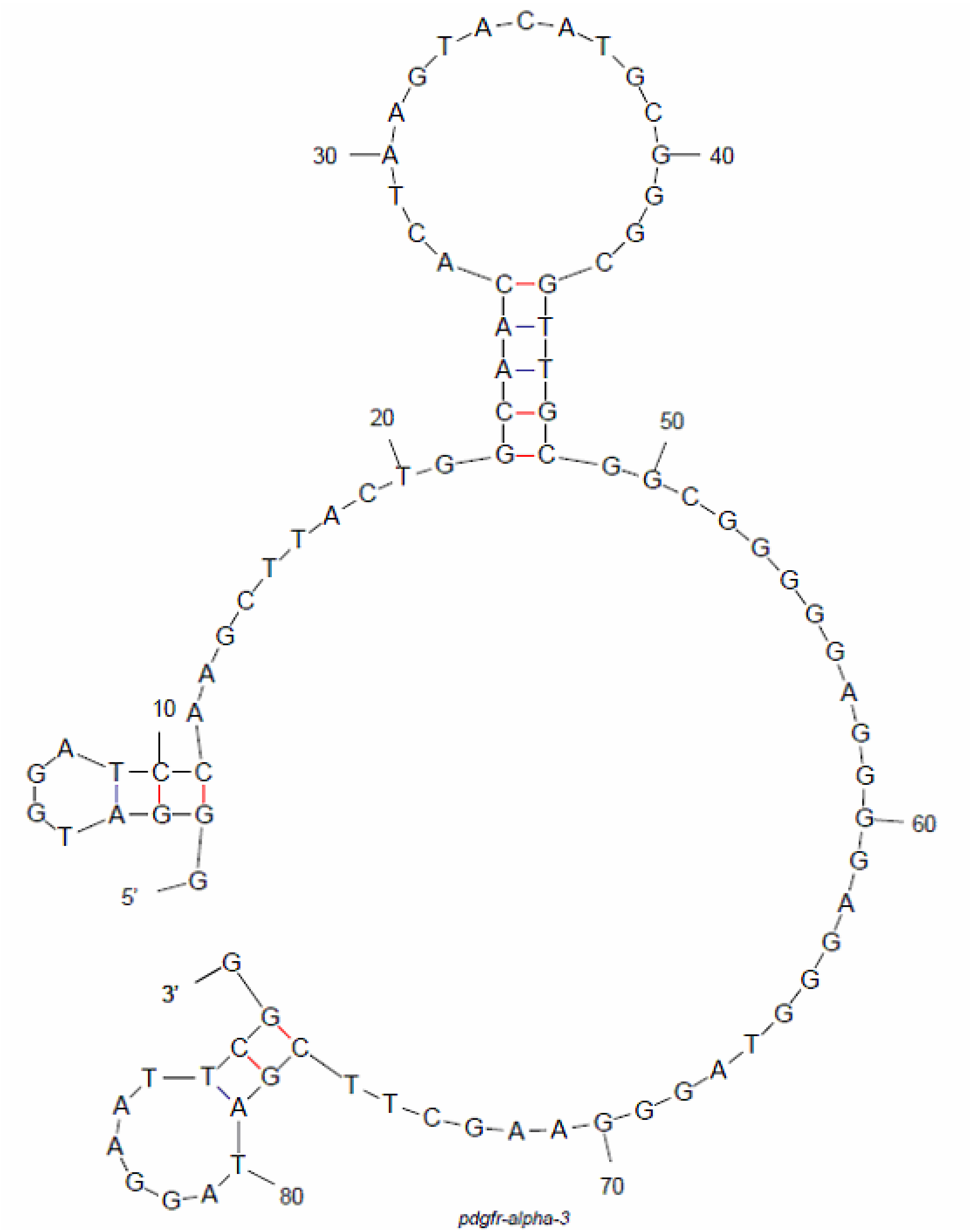


Figure 25. The most favorable secondary structure for anti- PDGFRα4 aptamer

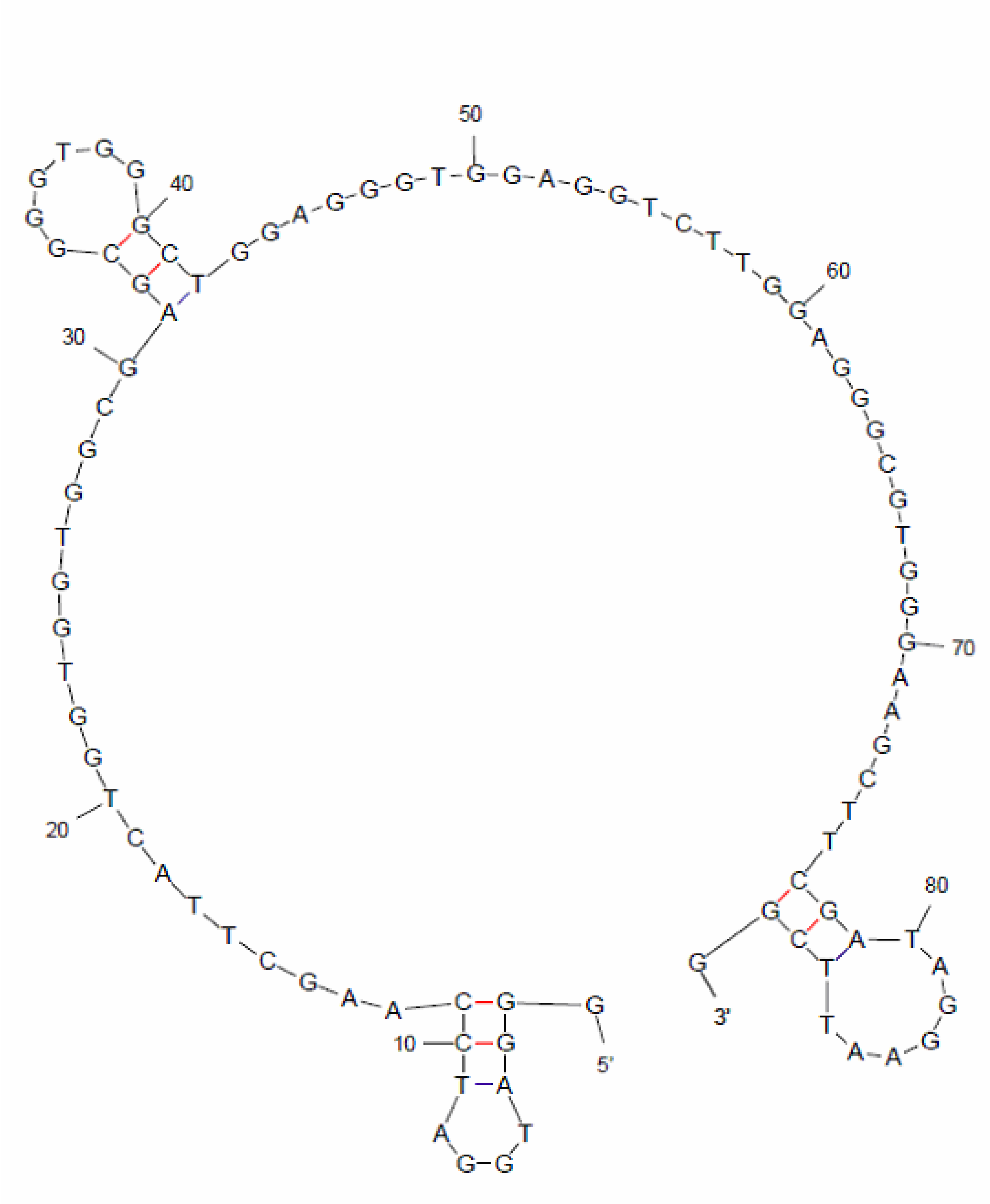


Figure 26. The most favorable secondary structure for anti- PDGFRα5 aptamer

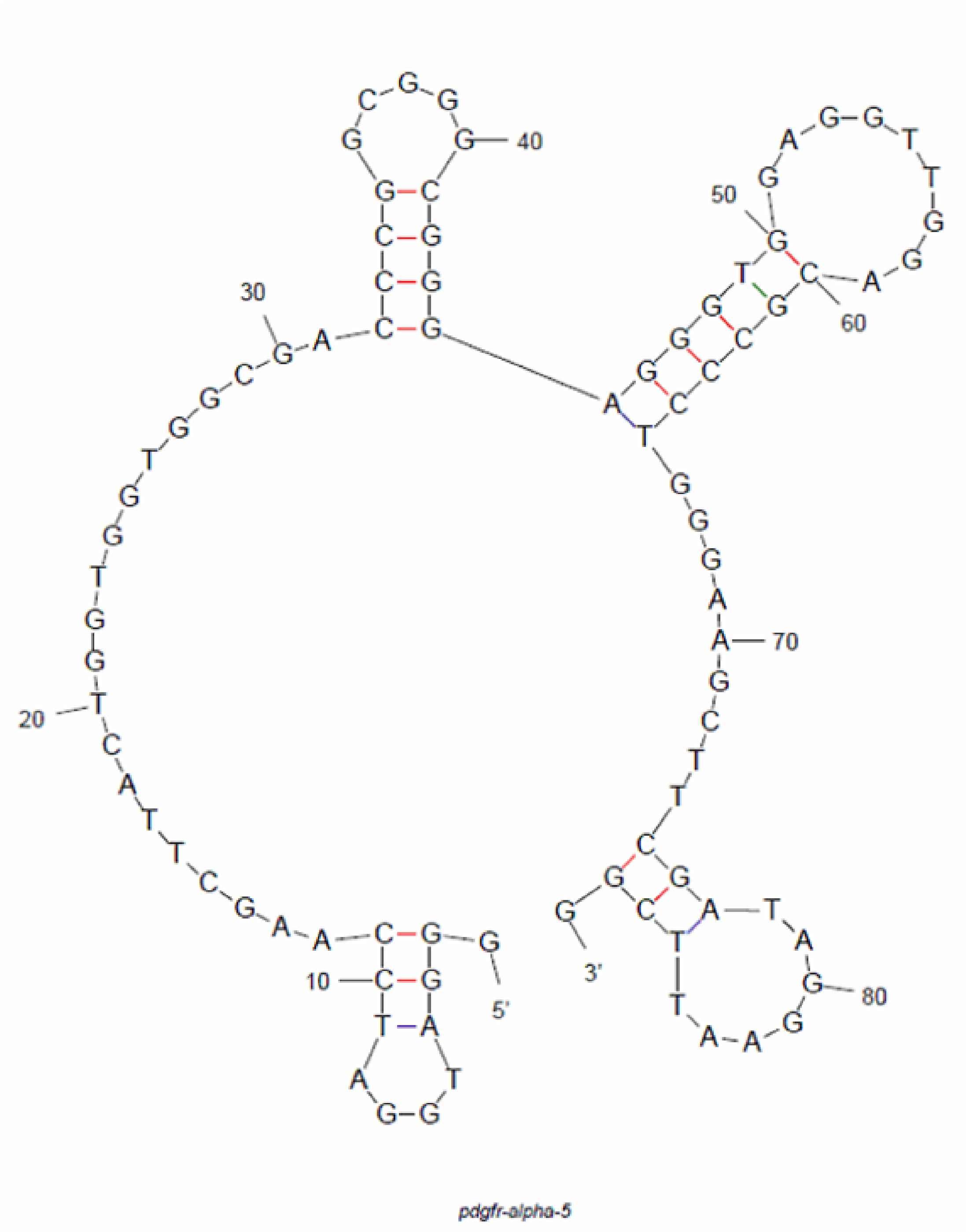


Figure 27. The most favorable secondary structure for anti- PDGFRα6 aptamer

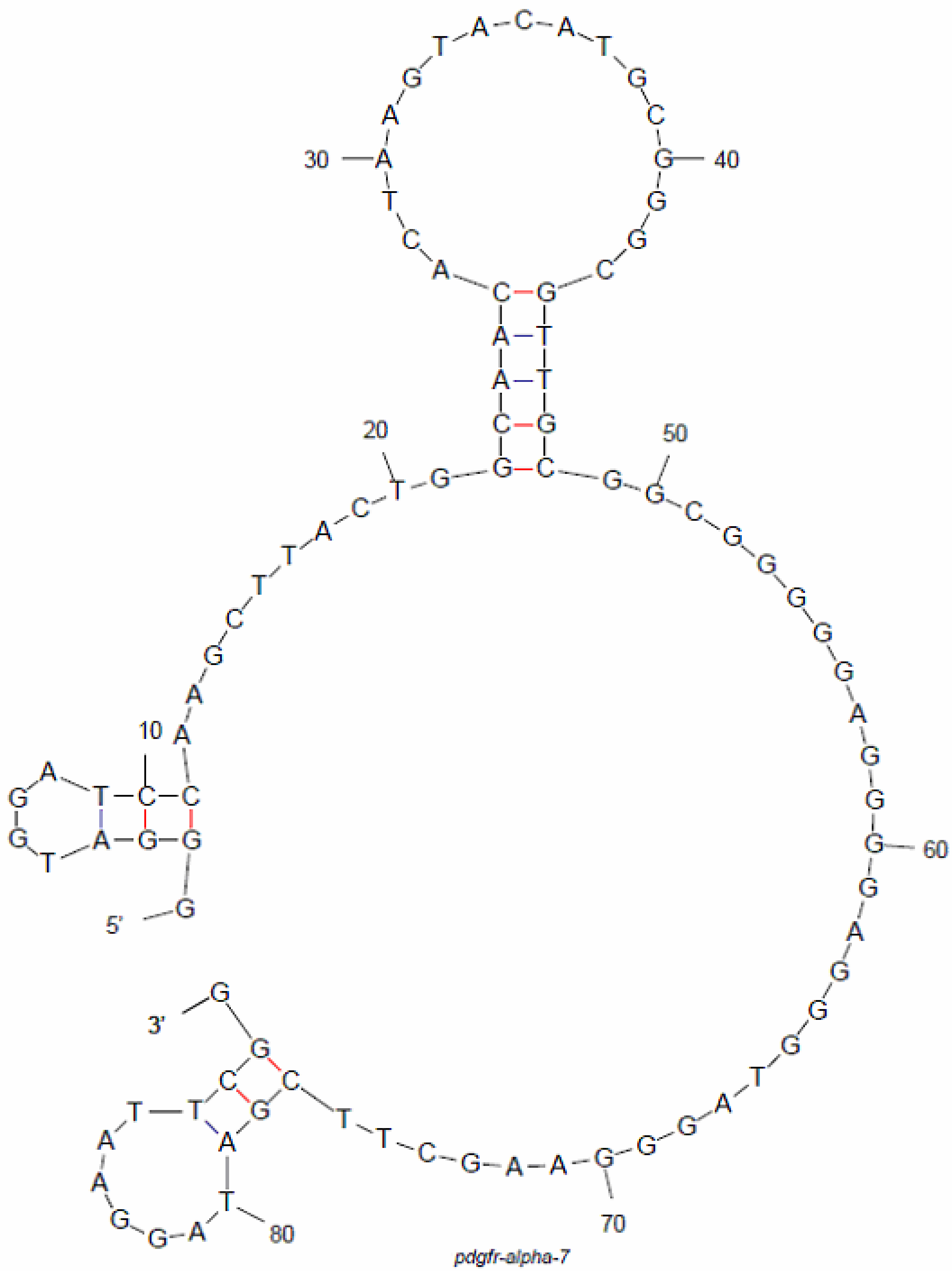


Figure 28. The most favorable secondary structure for anti- PDGFRα7 apamer

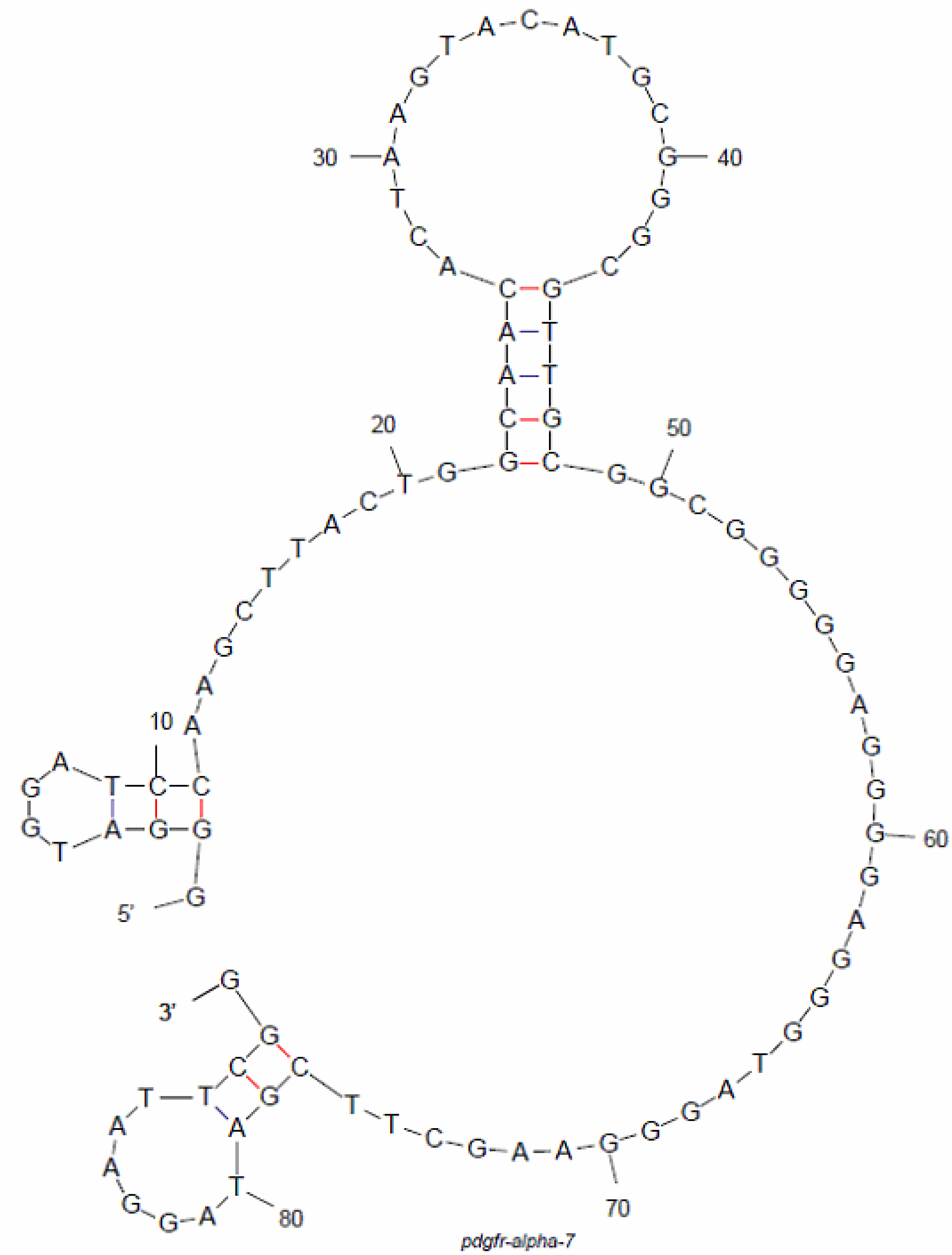


Figure 29. The most favorable secondary structure for anti- PDGFRα8 aptamer

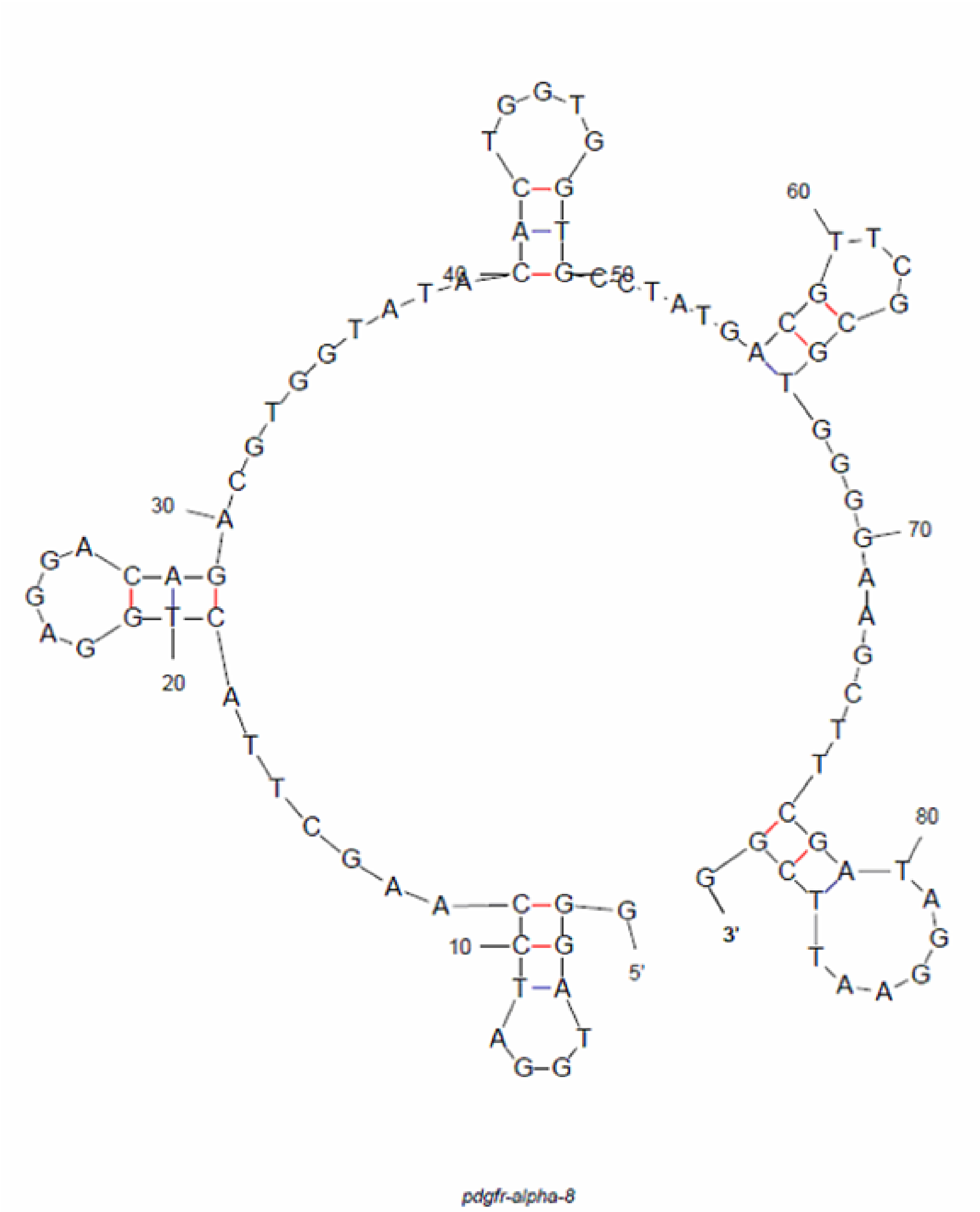


Figure 30. The most favorable secondary structure for anti- PDGFRα9 aptamer

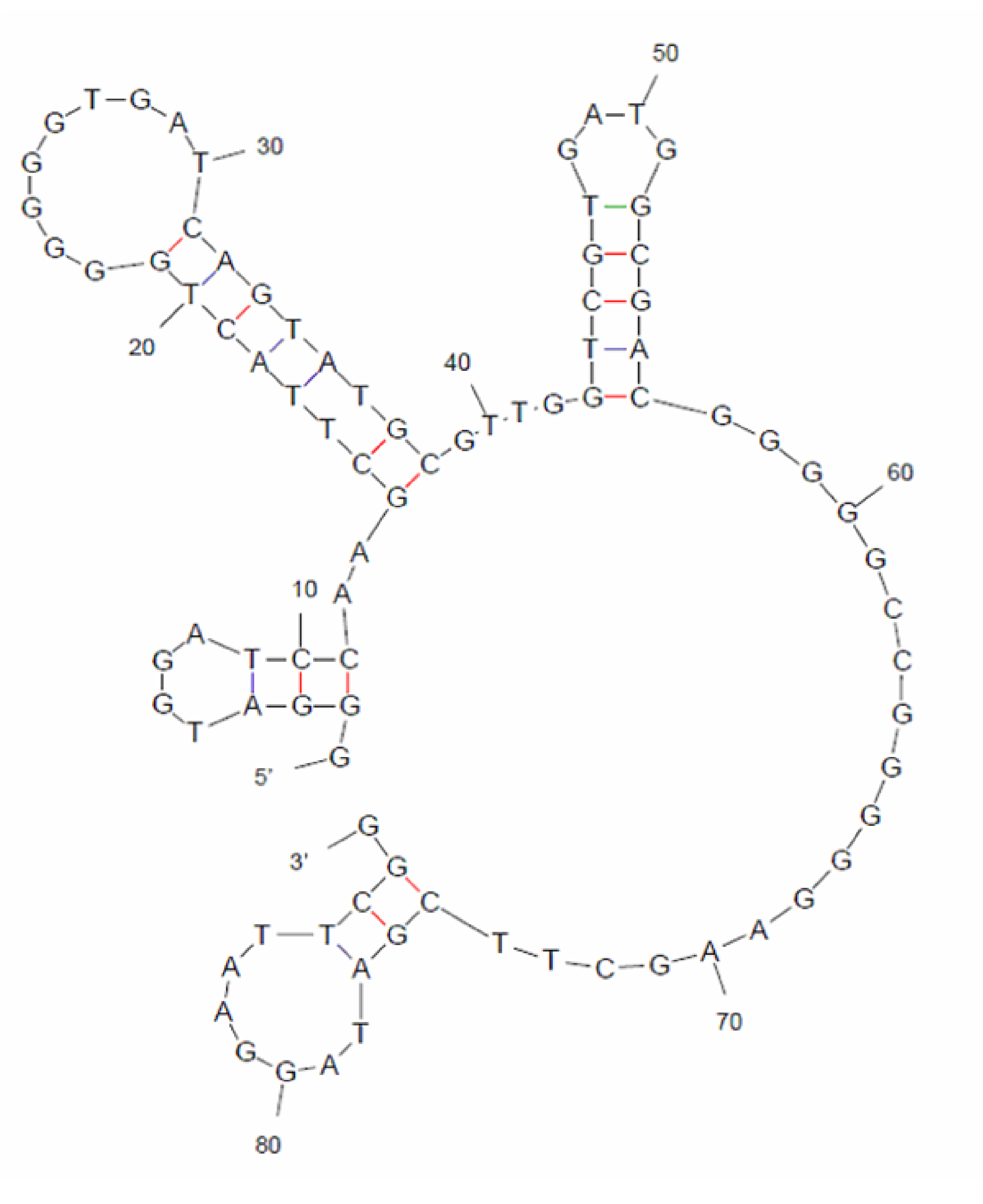


Figure 31. The most favorable secondary structure for anti- PDGFRα10 aptamer

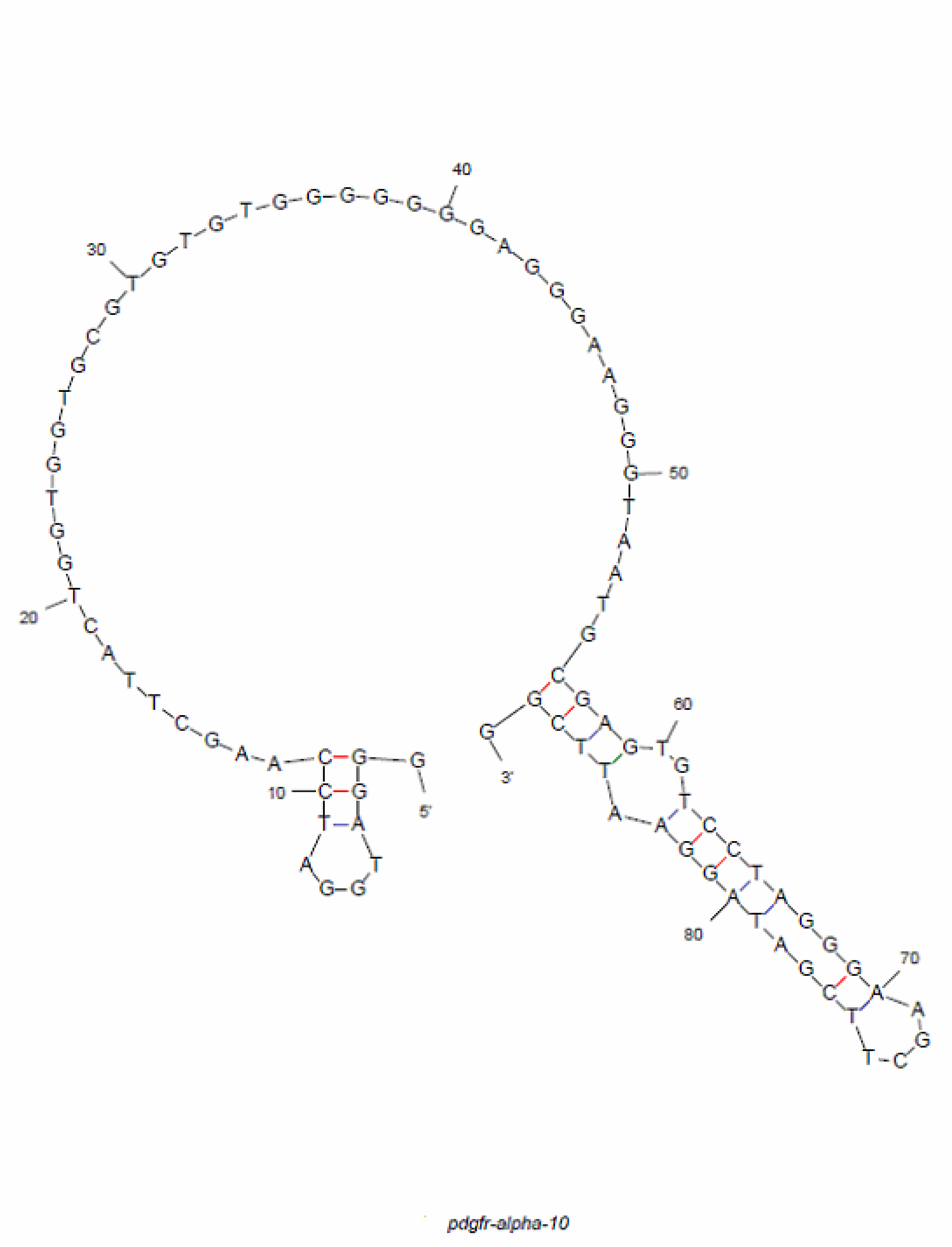


Figure 32. The most favorable secondary structure for anti- PDGFR β1 aptamer

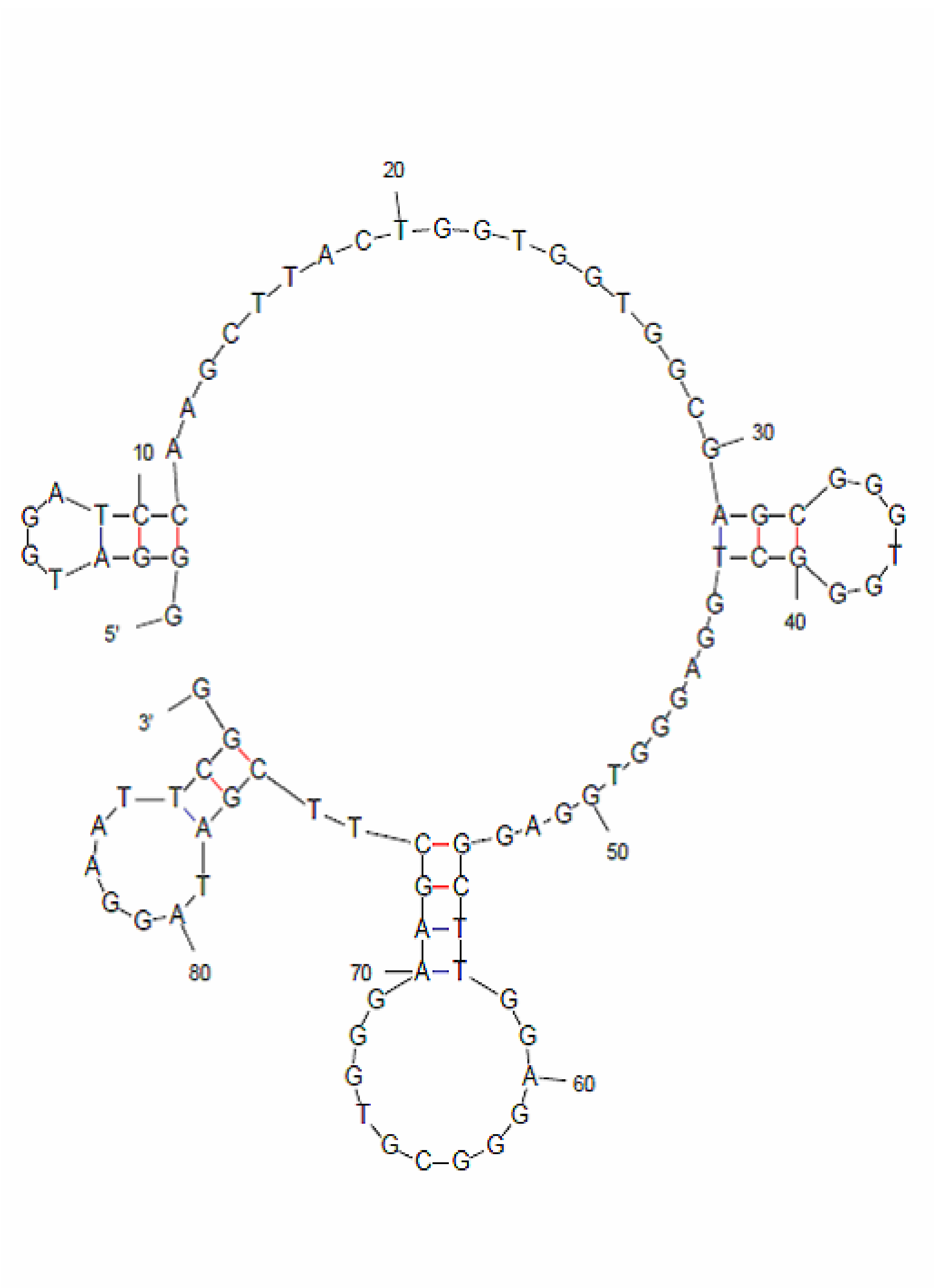


Figure 33. The most favorable secondary structure for anti- PDGFR β2 aptamer

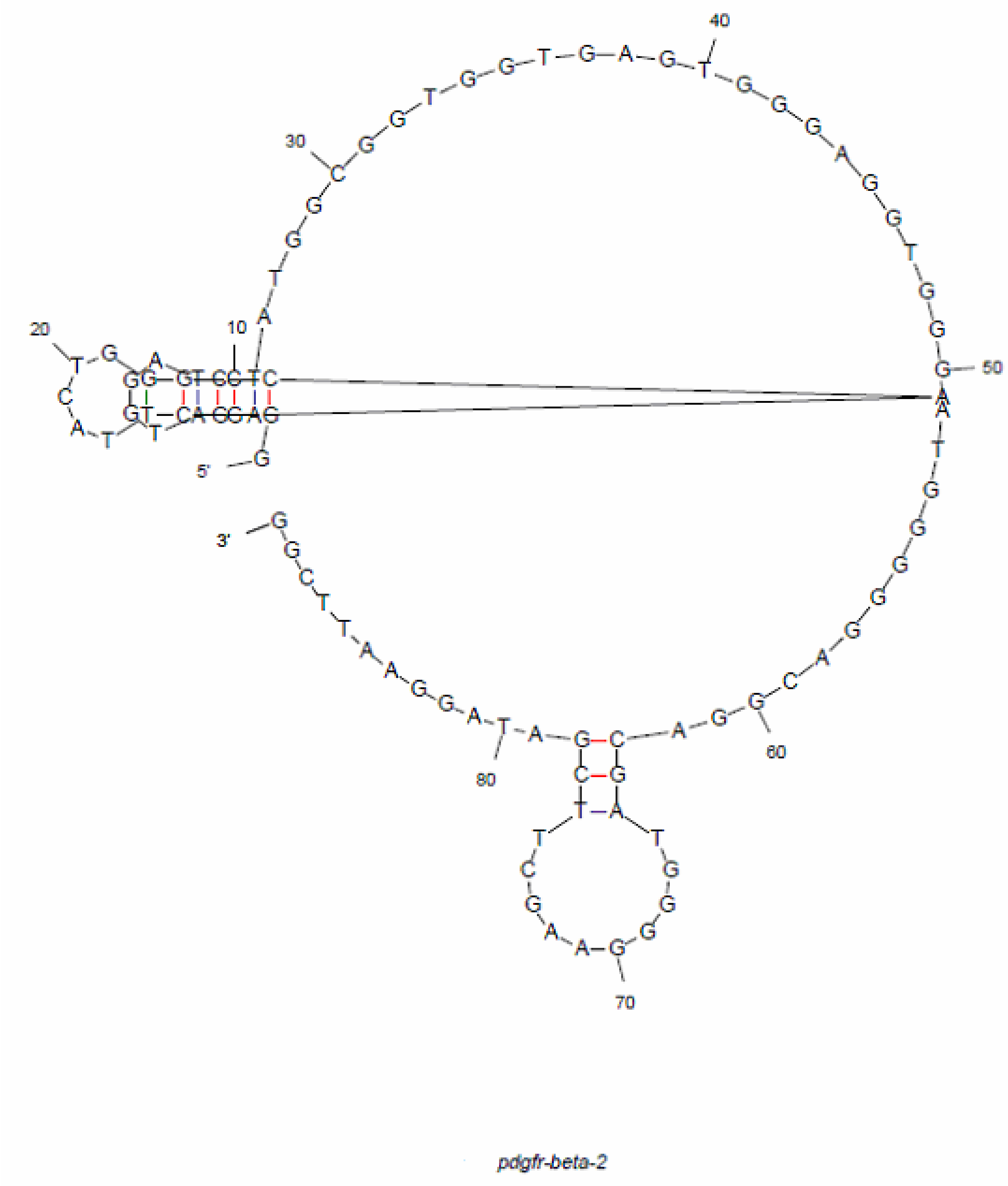


Figure 34. The most favorable secondary structure for anti- PDGFR β3 aptamer

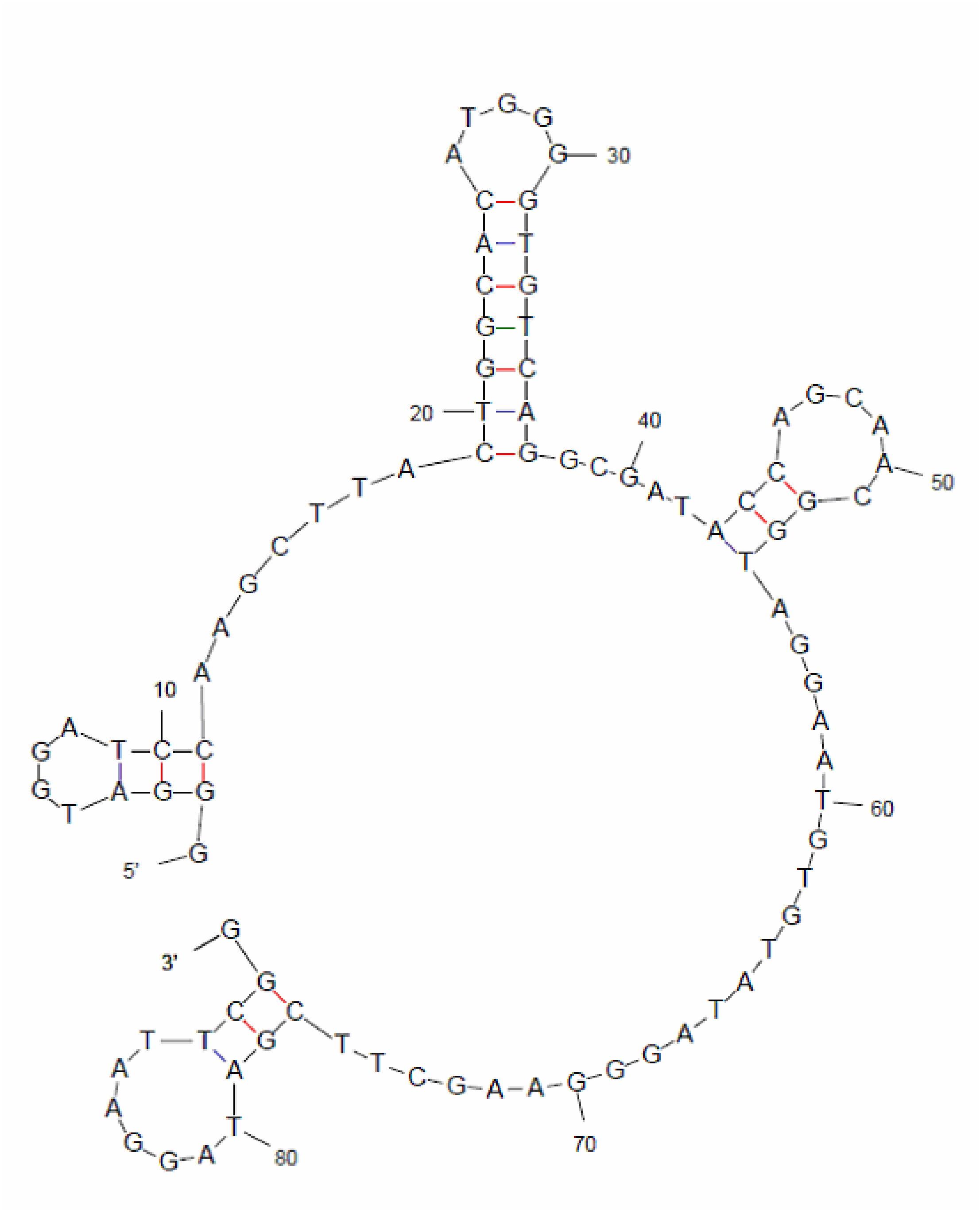


Figure 35. The most favorable secondary structure for anti- PDGFR β4 aptamer

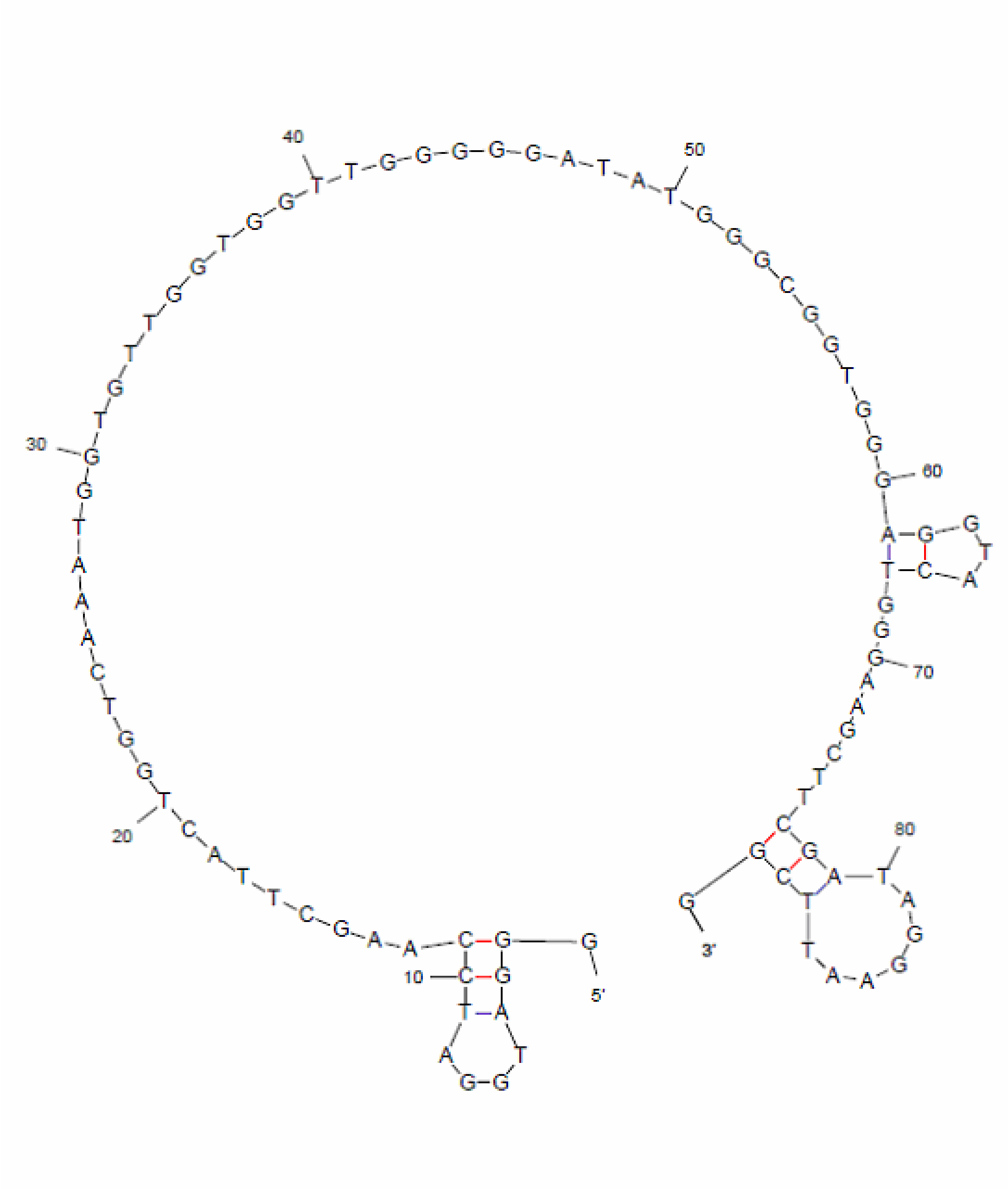


Figure 36. The most favorable secondary structure for anti- PDGFR β5 aptamer

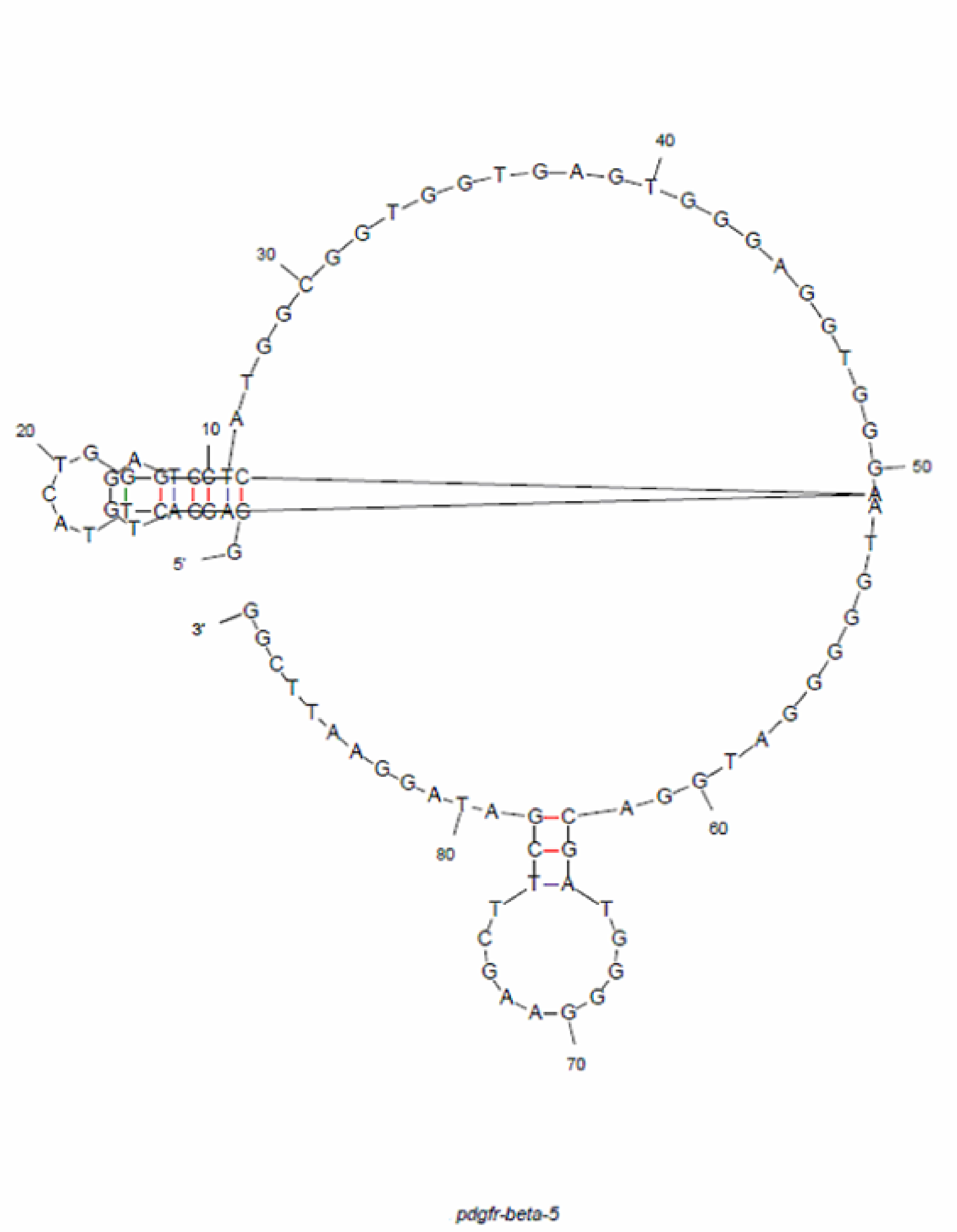


Figure 37. The most favorable secondary structure for anti- PDGFR β6 aptamer

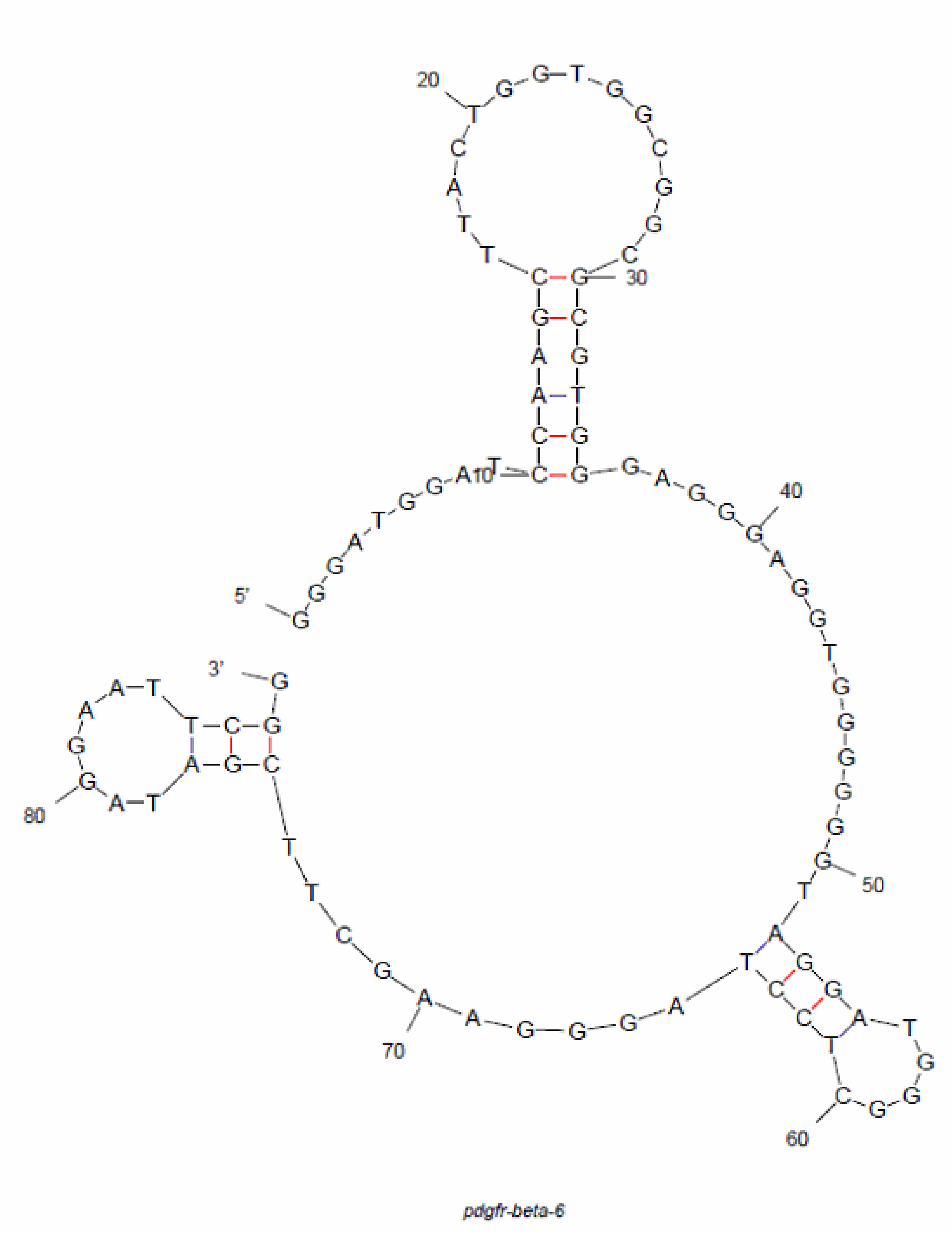
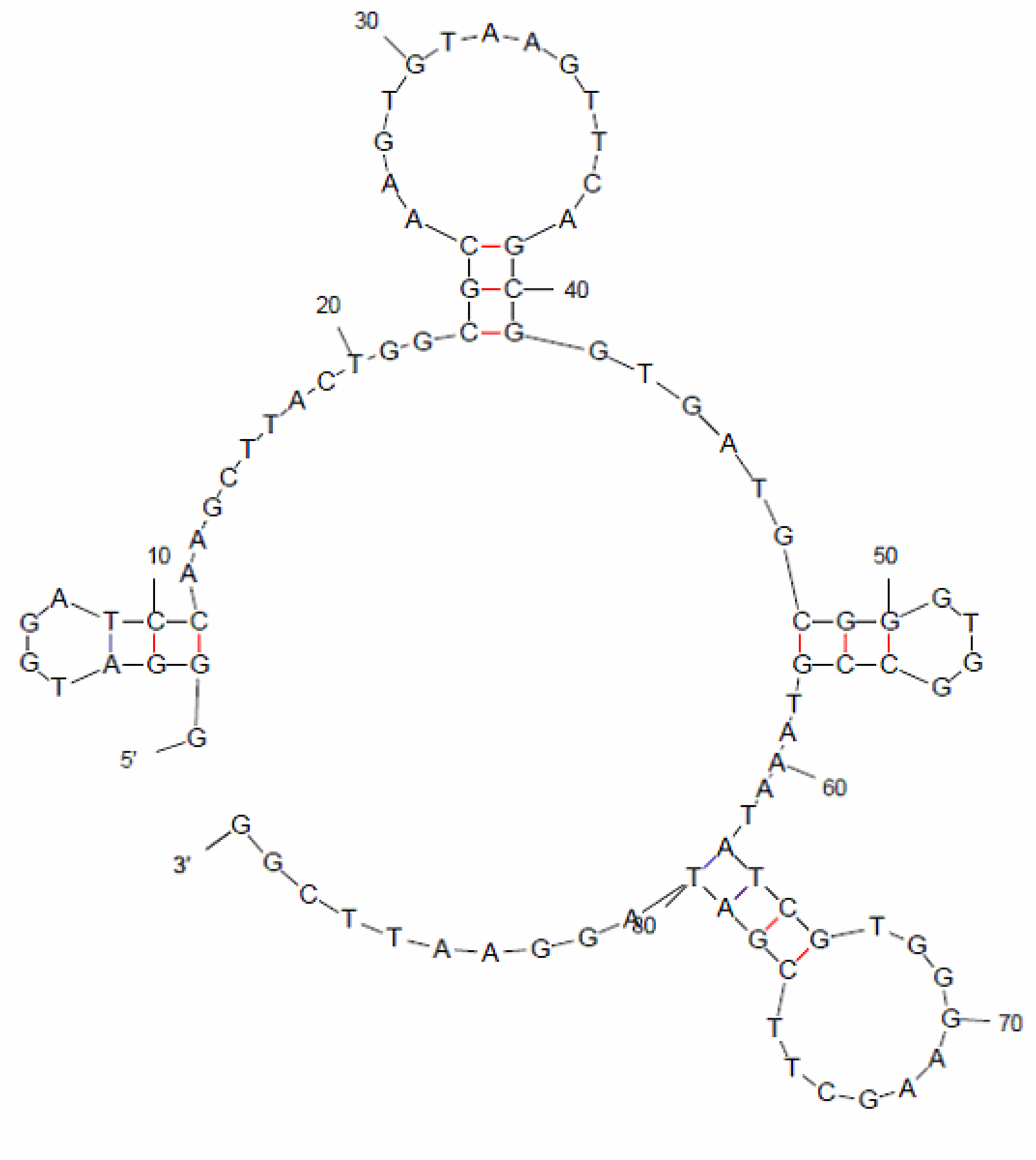


Figure 38. The most favorable secondary structure for anti- PDGFR β7 aptamer



pdgfr-beta-7

Figure 39. The most favorable secondary structure for anti- PDGFR β8 aptamer

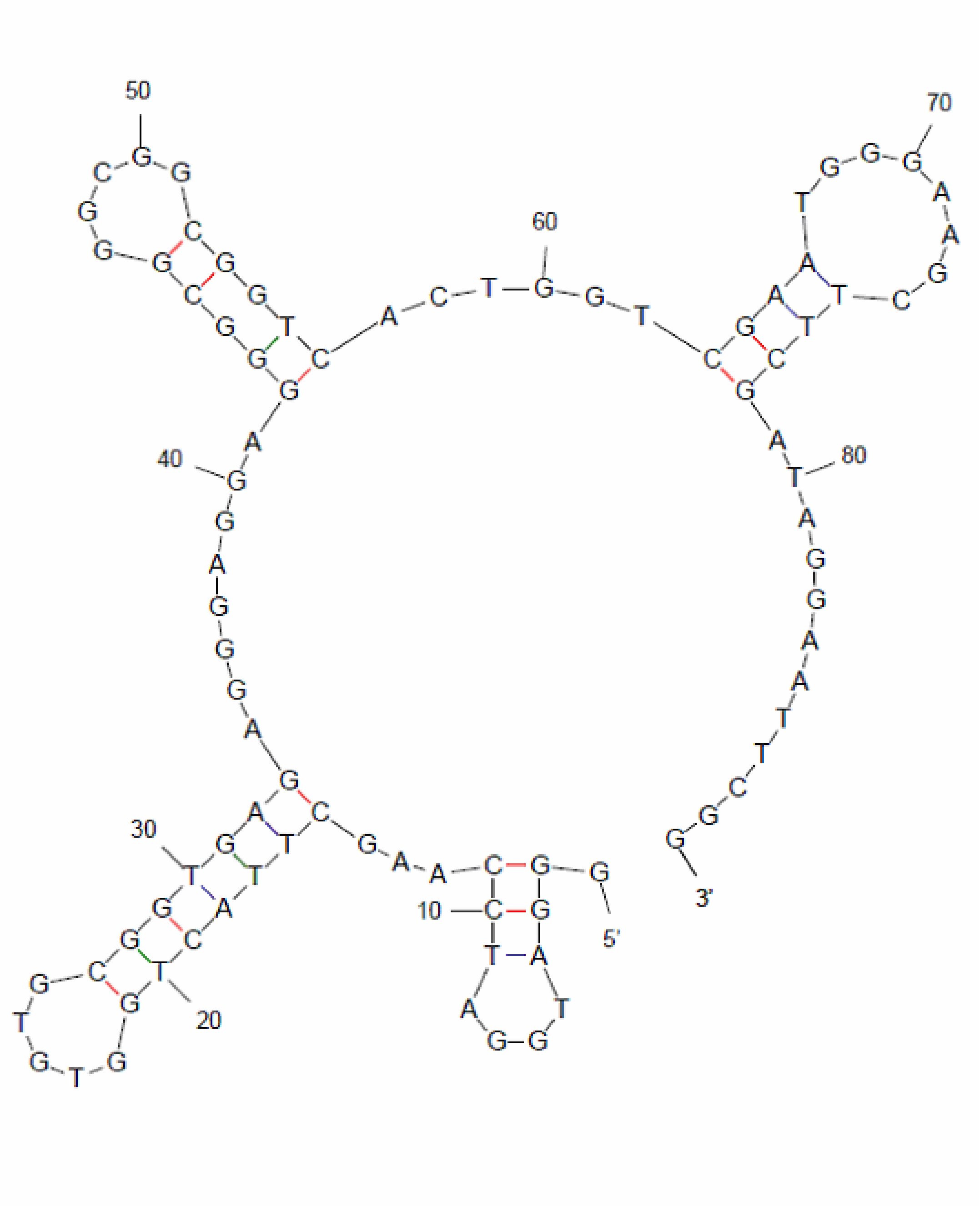


Figure 40. The most favorable secondary structure for anti- PDGFR β9 aptamer

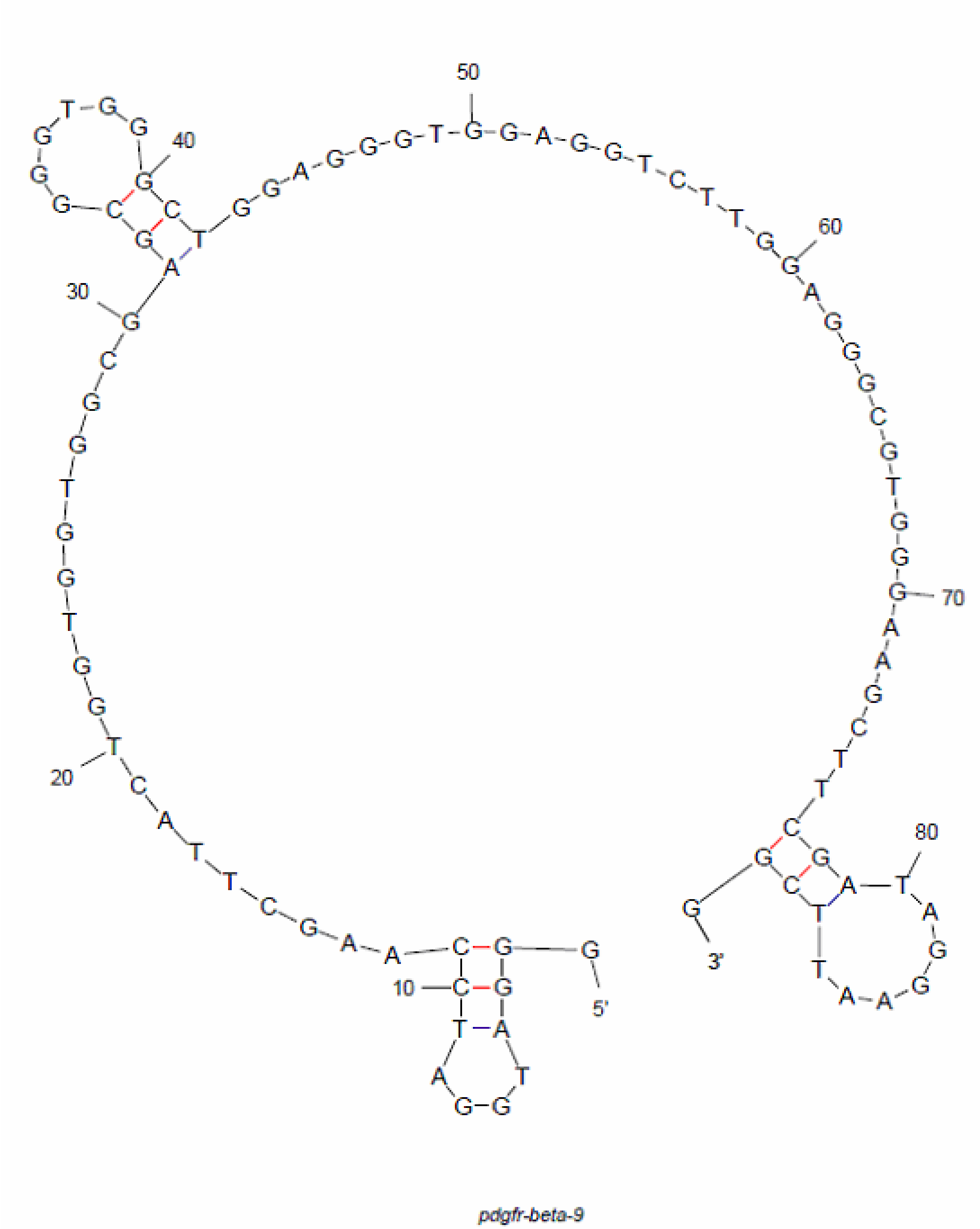


Figure 41. The most favorable secondary structure for anti- PDGFR β10 aptamer

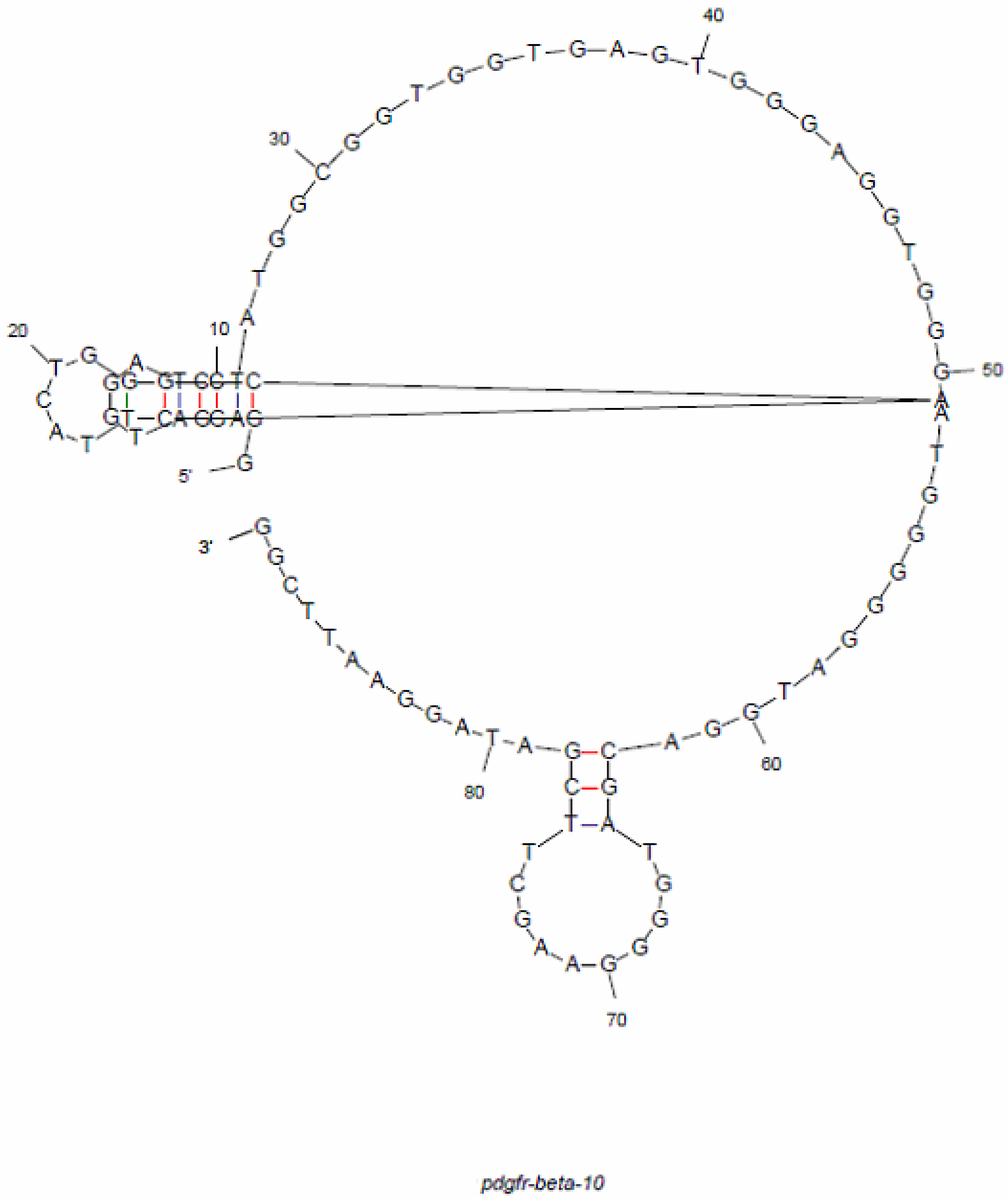


Figure 42. The most favorable secondary structure for anti- PDGFR β11 aptamer

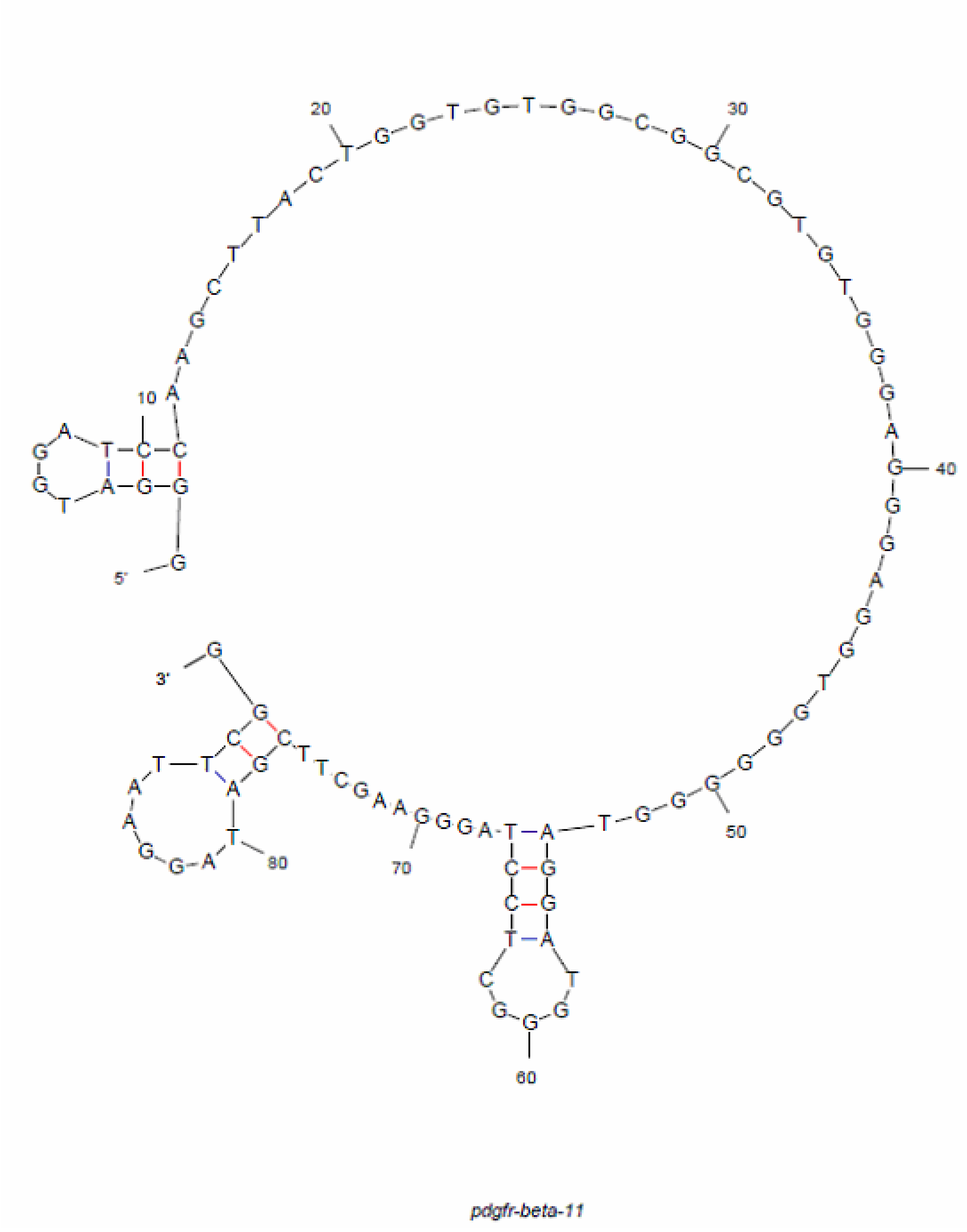


Figure 43. The most favorable secondary structure for anti- PDGFR β12 aptamer

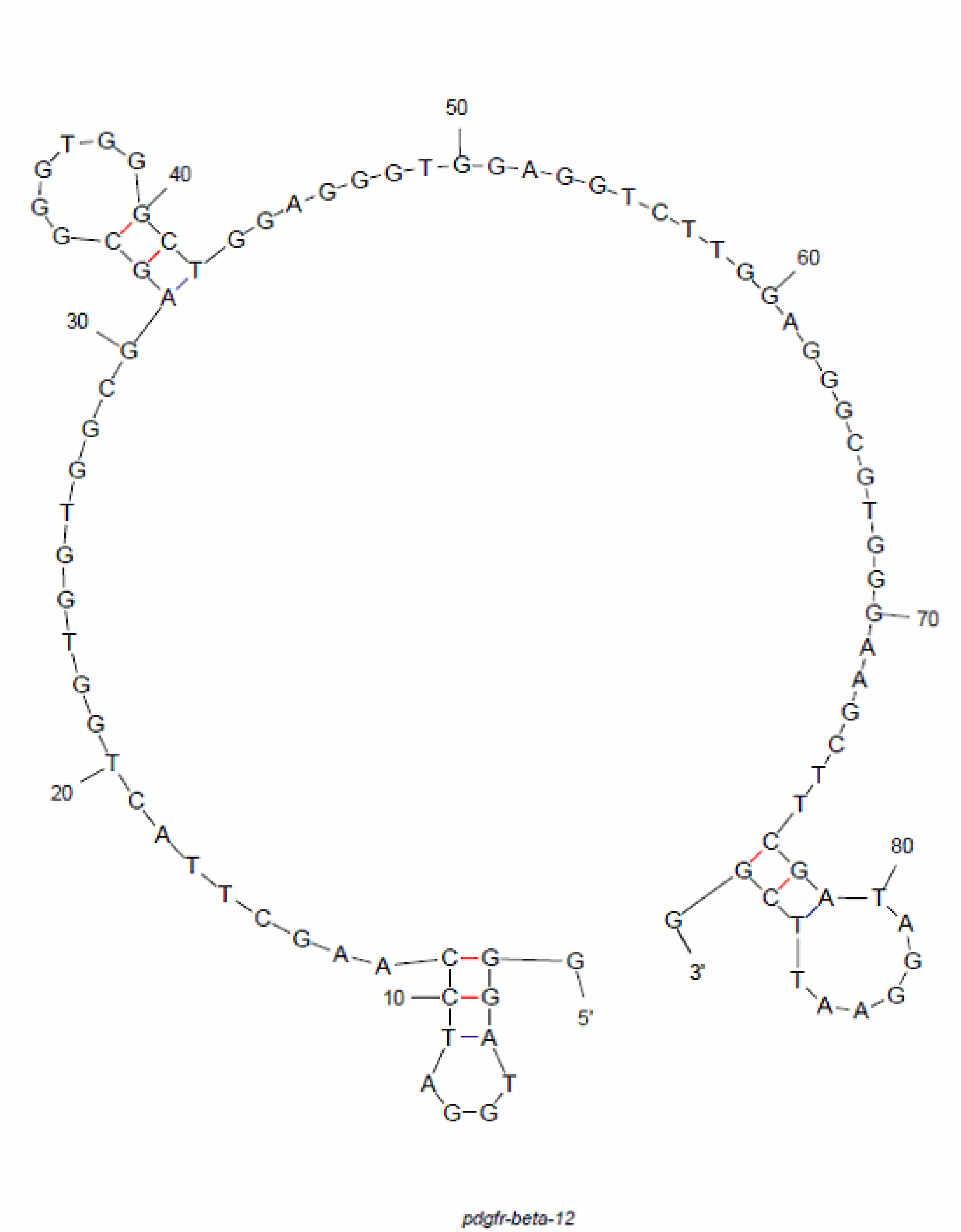


Figure 44. The most favorable secondary structure for anti- PDGFR β13 aptamer

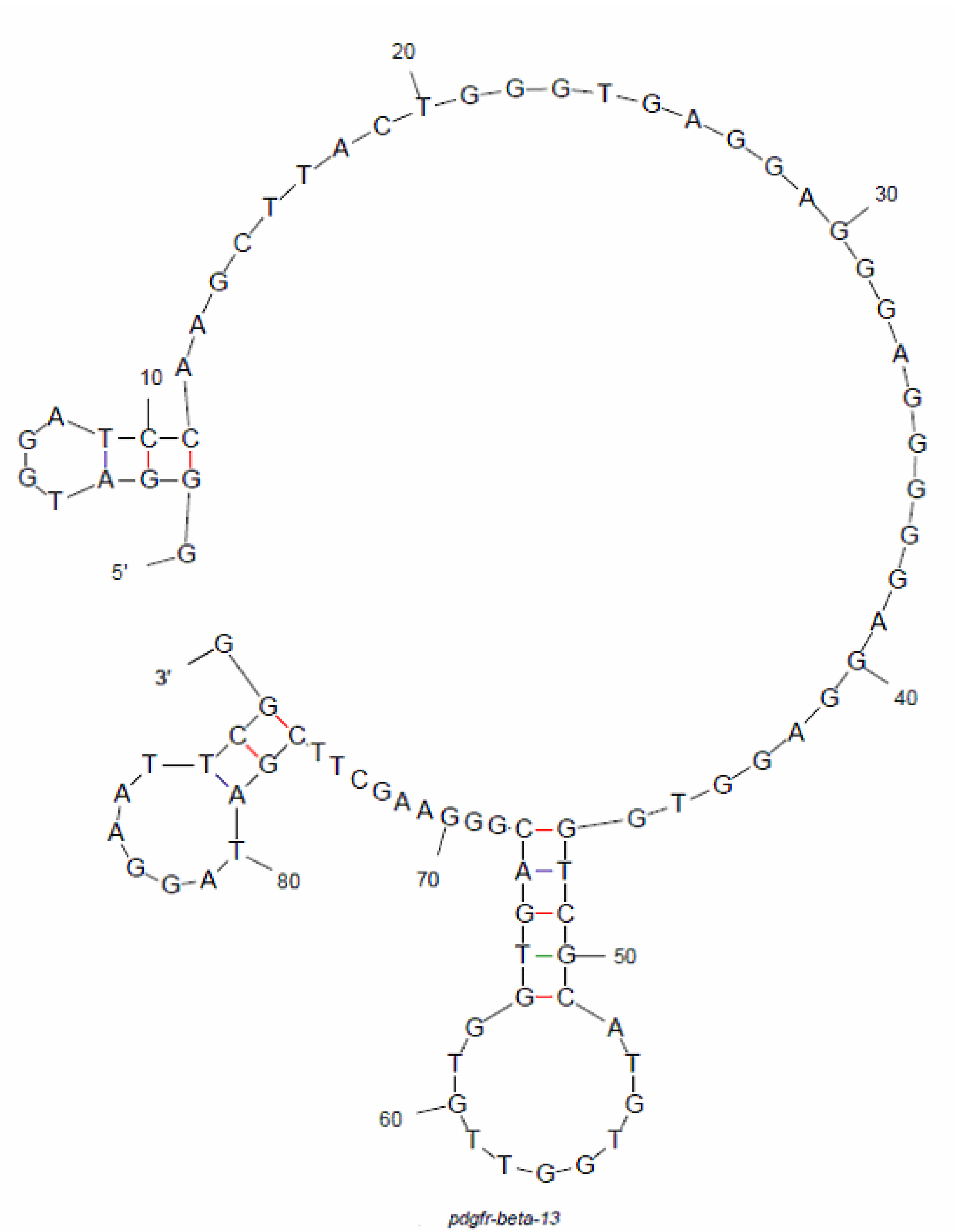
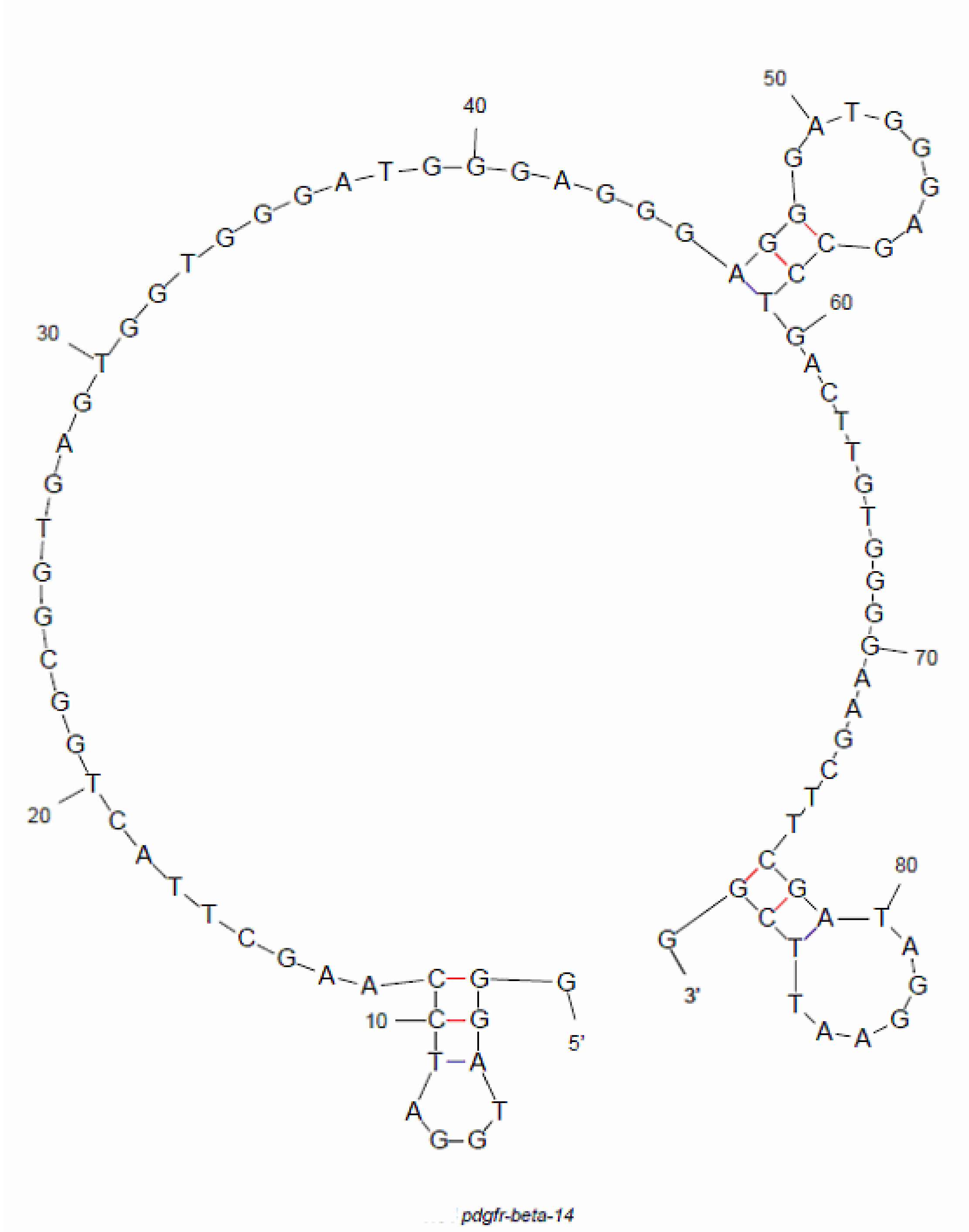


Figure 45. The most favorable secondary structure for anti- PDGFR β14 apamer



4.6 Predicting the G- quadruplexes in nucleotide sequences

G- quadraplexes of the selected aptamers can be detected by QGRS Mapper program; which is a web- based server for predicting G- quadruplexes in nucleotide sequences. It was used to examine the selected anti-PDGFRs aptamers for any G-quadruplex. It is available at http://bioinformatics.ramapo.edu/QGRS/analyze.php, and the results are summarized in Tables 8 and 9 respectively.

Table 8. Quadruplex-forming G-rich sequences (QGRS) of anti- PDGFRα aptamers using the QGRS mapper

Name of the selected aptamer	length	Position	QGRS	G-score
PDGFRα1	1	25	GGGATGGATCCAAGCTTACTGGTGG	9
	39	30	GGGAGGTGGGGGGTAGGATGGGCTCCTGGG	40
PDGFRα2	2	25	GGATGGATCCAAGCTTACTGGTAGG	10
	31	11	GGCGGTGGTGG	21
	43	18	GGGATGGGATGGGATGGG	42
	65	26	GGTGGGAAGCTTCGATAGGAATTCGG	12
PDGFRα3	53	18	GGGGAGGGGAGGGTAGGG	42
PDGFRα4	1	28	GGGATGGATCCAAGCTTACTGGTGGTGG	11
	35	14	GGTGGGCTGGAGGG	21
	53	18	GGTCTTGGAGGGCGTGGG	19
PDGFRα5	1	28	GGGATGGATCCAAGCTTACTGGTGGTGG	11
	35	14	GGCGGGCGGGAGGG	21
	50	18	GGAGGTTGGACGCCCTGG	15
PDGFRα6	21	27	GGGGGGGGTGGATCGCGGGAGGGCGGG	36
PDGFRα7	53	18	GGGGAGGGGAGGGTAGGG	42
PDGFRα8	21	25	GGAGGACAGACGTGGTATACACTGG	14
	66	24	GGGGAAGCTTCGATAGGAATTCGG	10
PDGFRα9	1	26	GGGATGGATCCAAGCTTACTGGGGGG	10
	56	12	GGGGGCCGGGGG	20
PDGFRα10	1	25	GGGATGGATCCAAGCTTACTGGTGG	9
	35	16	GGGGGGGAGGGAAGGG	41

Table 9. Quadruplex-forming G-rich sequences (QGRS) of anti- PDGFRβ aptamers using the QGRS mapper

Name of the selected aptamer	length	Position	QGRS	G-score
PDGFRβ1	1	28	GGGATGGATCCAAGCTTACTGGTGGTGG	11
	34	30	GGGTGGGCTGGAGGGTGGAGGCTTGGAGGG	31
PDGFRβ2	22	14	GGCTATGGCGGTGG	18
	41	29	GGGAGGTGGGATGGGGGACGGACGATGGG	37
PDGFRβ3	21	18	GGCACATGGGGTGTCAGG	16
PDGFRβ4	21	19	GGTCAAATGGTGTTGGTGG	16
	43	28	GGGGATATGGGCGGTGGGAGGTACTGGG	39
PDGFRβ5	21	14	GGCTATGGCGGTGG	18
	35	29	GGGAGGTGGGATGGGGGATGGACGATGGG	37
PDGFRβ6	1	28	GGGATGGATCCAAGCTTACTGGTGGCGG	11
	34	17	GGGAGGGAGGTGGGGGG	38
	53	29	GGATGGGCTCCTAGGGAAGCTTCGATAGG	13
PDGFRβ7	41	29	GGTGATGCGGGTGGCCGTAAATATCGTGG	10
PDGFRβ8	1	29	GGGATGGATCCAAGCTTACTGGTGTGCGG	11
	35	14	GGGAGGAGGGCGGG	21
	50	20	GGCGGTCACTGGTCGAATGG	16
PDGFRβ9	1	28	GGGATGGATCCAAGCTTACTGGTGGTGG	11
	35	14	GGTGGGCTGGAGGG	21
	53	18	GGTCTTGGAGGGCGTGGG	19
PDGFRβ10	22	14	GGCTATGGCGGTGG	18
	41	29	GGGAGGTGGGATGGGGGATGGACGATGGG	37
PDGFRβ11	1	27	GGGATGGATCCAAGCTTACTGGTGTGG	11
	36	17	GGGAGGGAGGTGGGGGG	38
	55	29	GGATGGGCTCCTAGGGAAGCTTCGATAGG	13
PDGFRβ12	1	28	GGGATGGATCCAAGCTTACTGGTGGTGG	11
	35	14	GGTGGGCTGGAGGG	21
	53	18	GGTCTTGGAGGGCGTGGG	19

PDGFRβ13	**1**	**28**	**GGGATGGATCCAAGCTTACTGGGTGAGG**	**11**
	34	**11**	**GGGGGAGGAGG**	**21**
	46	**24**	**GGTCGCATGTGGTTGTGGTGACGG**	**17**
PDGFRβ14	**6**	**27**	**GGATCCAAGCTTACTGGCGGTGAGTGG**	**9**
	34	**16**	**GGGATGGGAGGGAGGG**	**41**
	67	**24**	**GGGGAAGCTTCGATAGGAATTCGG**	**10**

The G-score was calculated according to the number of Gs in the group and the length of the loop. The higher scores with the most stable G-quadruplexes are related to short loops and high number of G- bases in the guanine tetrads (Kinin O., et al., 2006).

5. Discussion

The SELEX process is an iterative methodology designed to create target-specific aptamers from a combinatorial library that bind to a variety of molecular entities with high affinity and specificity. The aim of the SELEX experiment described here is to identify ssDNA aptamers against the tyrosine kinase domain of both PDGFRα and PDGFRβ. These enzymes were selected in view of their importance in the regulation of cell growth and the fact that abnormalities in these proteins can lead to constitutive activation of PDGFRs that may have pathological consequences (Funa et al., 2003; Swick et al., 2008).

The nucleic acid pools used for in vitro selection experiments typically contain around 90 bases with a randomized central core with 45 nucleotide in length, that can theoretically have 4^{45} different sequences, (where 4 is the number of nitrogenous bases in the random region), which represents about $1.24x10^{27}$ different copies.

Synthesis of the first ssDNA library was performed under the same conditions that were used during the trial extension reactions to ensure that the final pool has a complexity similar to the calculated one. In other words, when the small scale PCR reaction is optimized by using one hundred nanograms (approximately $2x10^{14}$ different sequences) of synthesized templates in 50 µl total volume, it was possible to amplify ten micrograms of synthesized template (approximately 2×10^{16} different sequences) in 5 ml of reaction mix. Similar approaches were considered for asymmetric PCR.

Recovery of ssDNA from asymmetric PCR product was performed by extraction and purification from low melting point agarose gel. This method showed good yields of ssDNA after extraction, enough to recover the largest amount of amplified ssDNA (up to 90% as described by Kurien and Scofield (2002)).

While there are multiple possible configurations for in vitro selection experiments, this study described a method for selection of ssDNA aptamers against Poly- His tag kinase

domain of PDGFR using magna-His Ni particles as an affinity separation method of aptamer- target complex. The affinity separation method using Poly- His tag ligand mediated magnetic system was described in many studies to isolate high affinity aptamers such as for prion proteins (Weiss et al., 1997) and MUC1 tumor marker (Rhie et al., 2003; Ferriera et al., 2006).

Anti-PDGFRs aptamers analysis:

It is not enough to know the structure of the sequences that were derived from the SELEX procedure; an analysis for these sequences is also important to predict the similarity and the differences among sequence and determine the conserved regions (if persent). This can be done by CLC main workbench which is an advanced software used for sequence analysis.

Figure 46 shows the alignment between anti- PDGFR α aptamers to predict the conserved sequences. This alignment was analyzed as a tree in Figure 47, to determine the similarity percentage between the anti- PDGFR α selected aptamers themselves, as well as with the anti- PDGFR β selected aptamers (See Figures 48 and 49). It is worth while also combing all the selected aptamers for PDGFRα and PDGFRβ in one alignment and tree (Figure 50 and 51).

Due to the similarity between PDGFRα and PDGFRβ in the kinase domain; it can be noticed from this alignment, the presence of the same sequences that were selected to both PDGFRα and PDGFRβ by the SELEX process. Obviously, there are sequences that were selected which bind to one target but not the other, which means that such aptamers can differentiate between these two homologous targets.

Figure 46. Alignment of anti-PDGFRα aptamers .

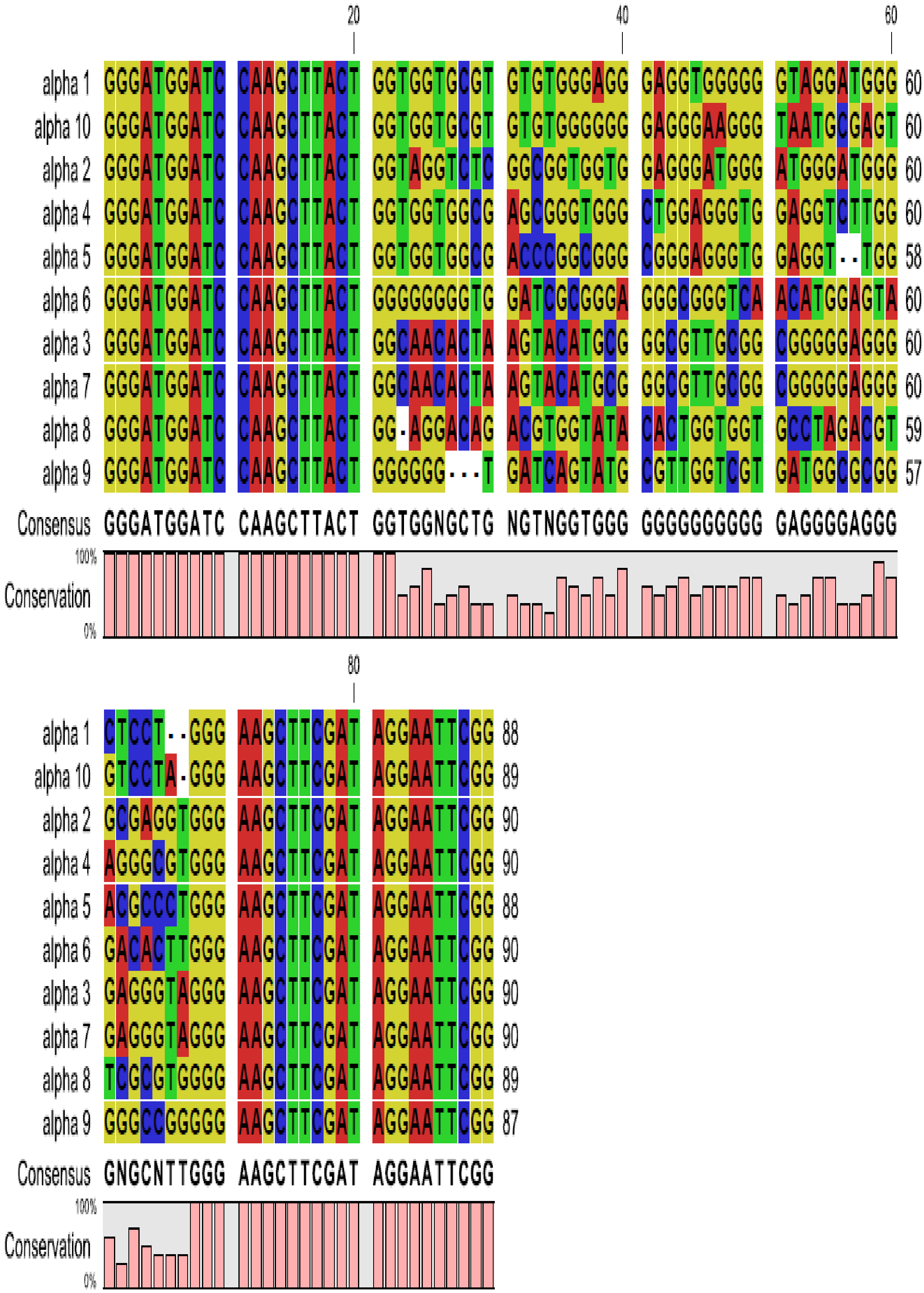


Figure 47. Tree analysis of anti-PDGFRα aptamers.

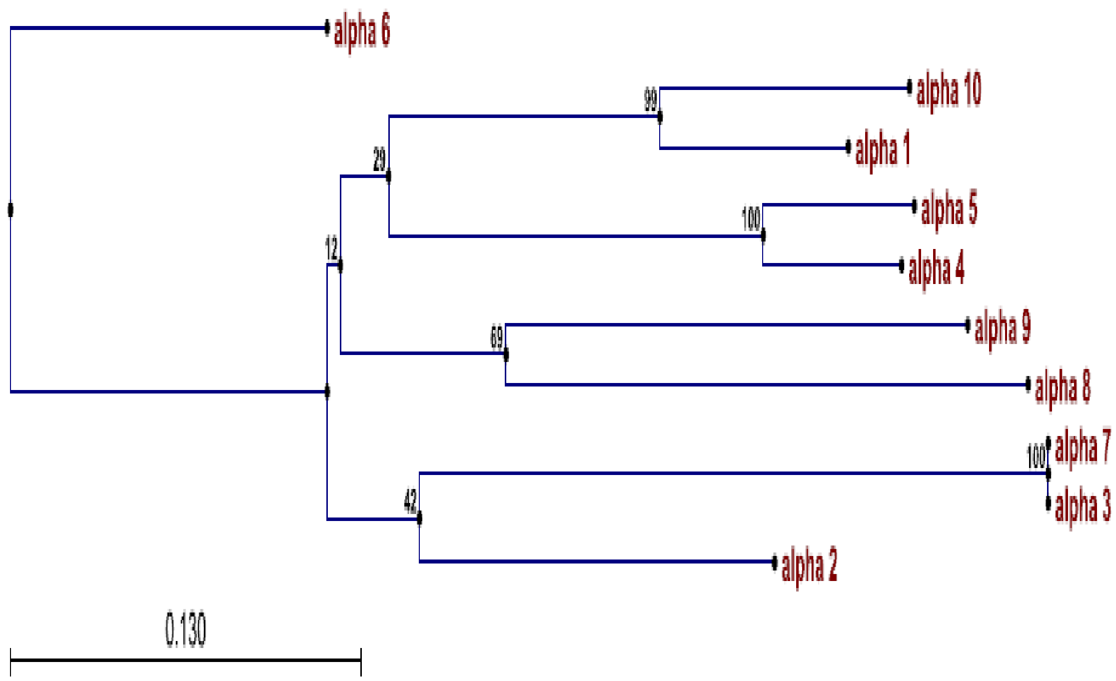


Figure 48. Alignment of anti-PDGFRβ aptamers.

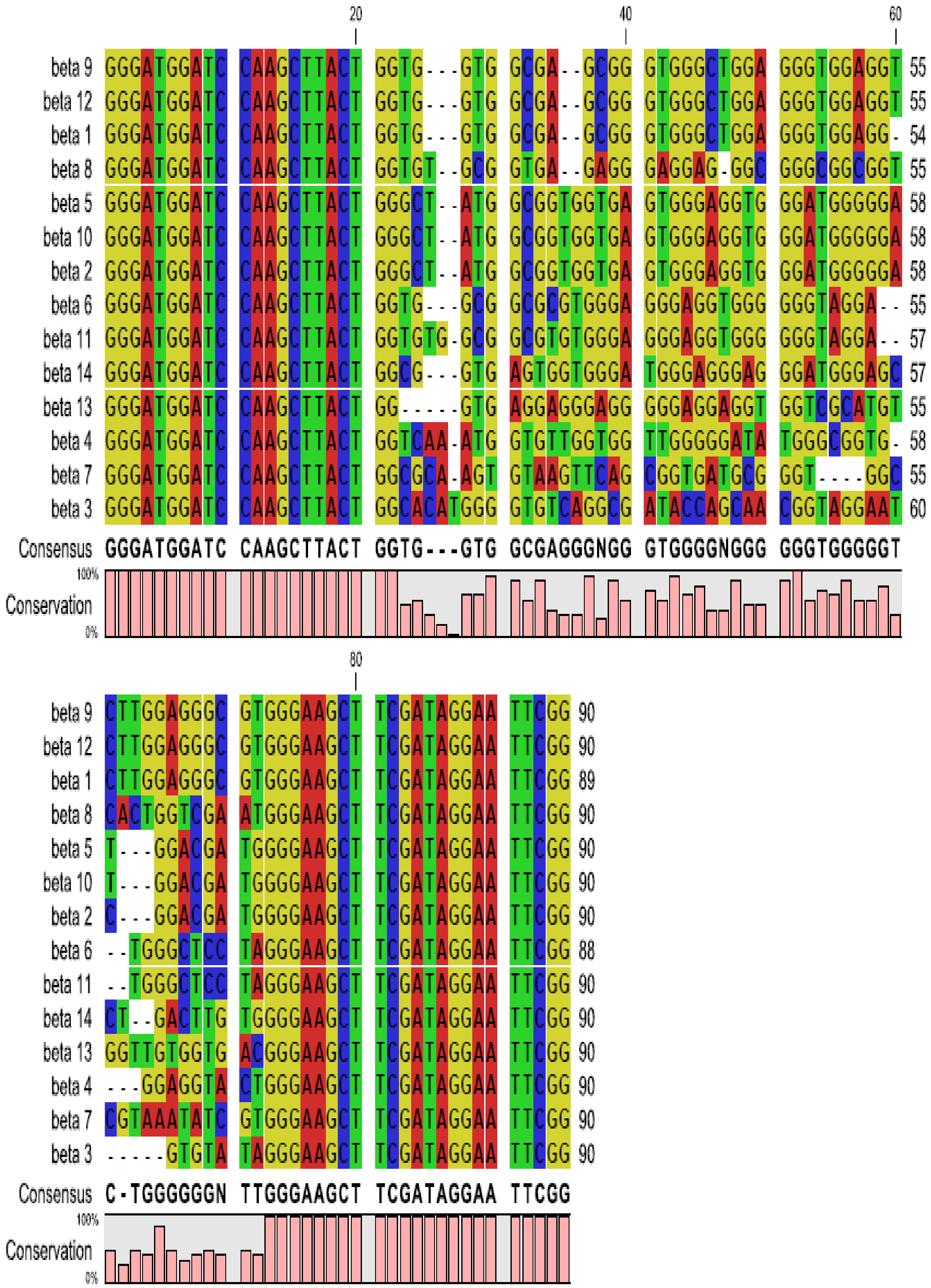


Figure 49. Tree analysis of anti-PDGFRβ aptamers.

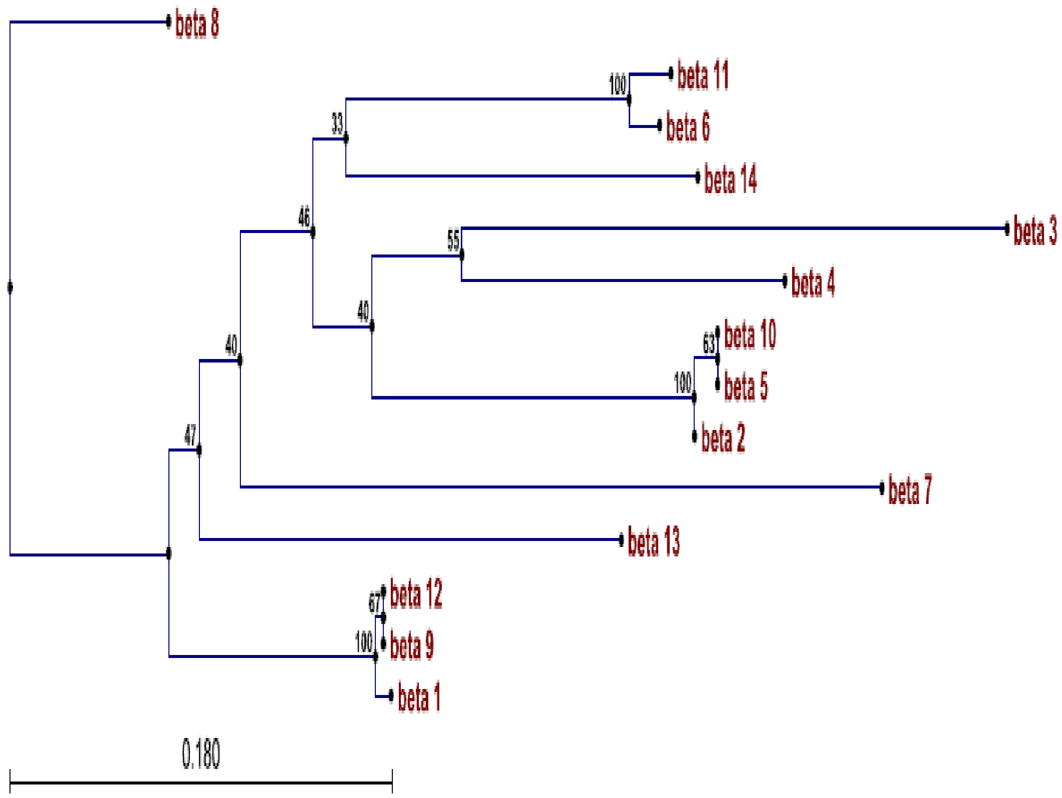


Figure 50. Alignment analysis for both anti-PDGFRα and anti-PDGFRβ aptamers.

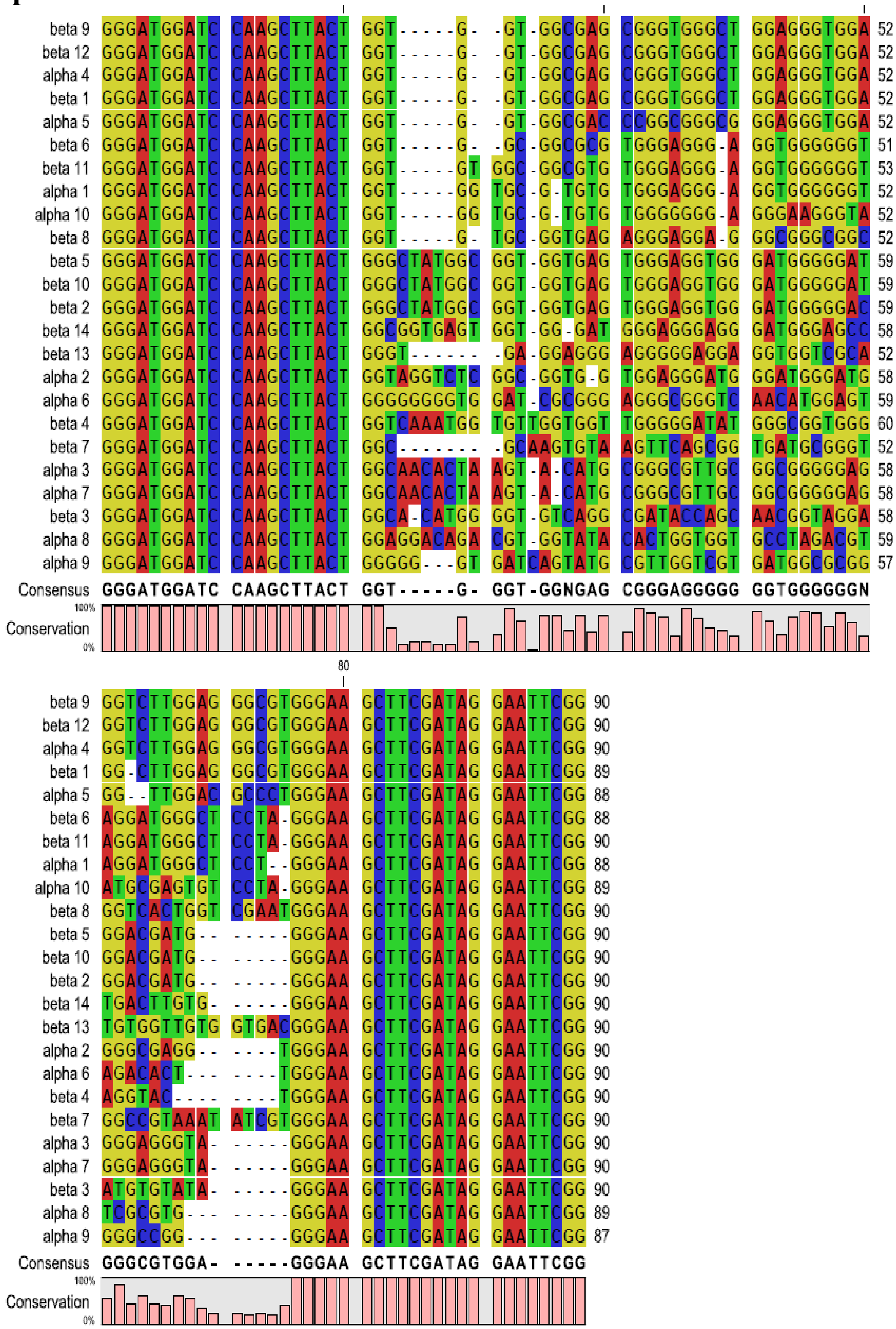
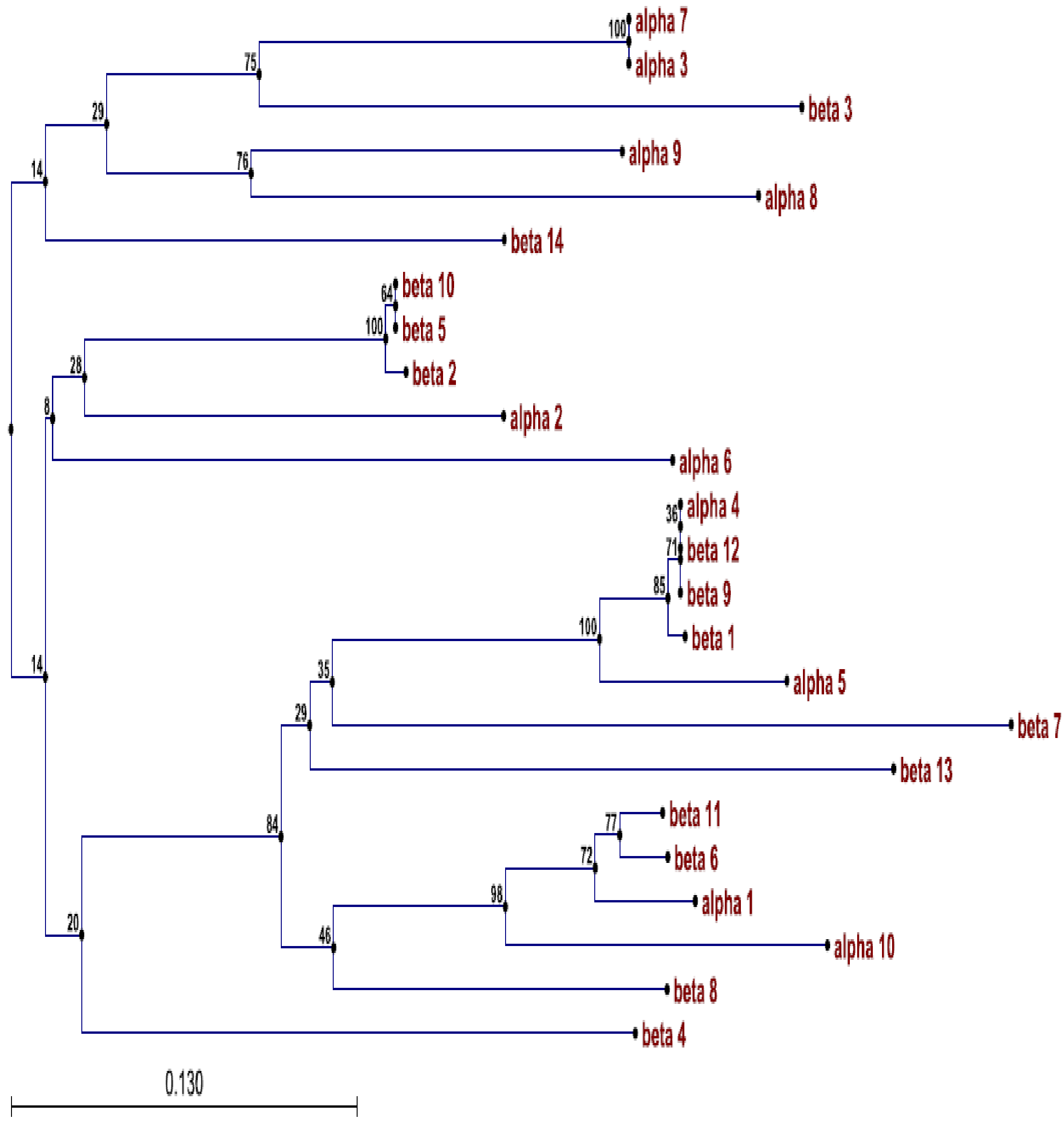


Figure 51. Tree analysis for both anti-PDGFRα and anti-PDGFRβ aptamers.



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Many common types of cancer, especially in their advanced stages, do not respond well to traditional chemotherapy or may acquire resistance to therapy. Novel agents that work by mechanisms that are different from existing therapies, particularly those that are able to target multiple pathways important for cancer cell survival, may therefore be useful in the treatment of advanced cancer.

Nucleic acid aptamers are emerging as a new class of targeted therapeutic agents and are the focus of a rapidly growing field of medical research. These small synthetic oligomers of DNA or RNA form stable three- dimensional structures and bind to defined molecular targets via shape specific recognition. Thus, in essence, their mechanism of action is similar to that of protein- based monoclonal antibodies (Choi et al., 2010). However, due to their smaller size and different chemical composition, aptamers have a number of potential advantages over antibodies. These include; ease of manufacture and storage, facile conjugation, better tumor penetration, more rapid systemic clearance and non-immunogenecity (Mayer, 2009; Choi et al., 2010).

In recent years, aptamers have been identified as potential treatment for a variety of diseases, including macular degeneration (Gragoudas et al., 2004), autoimmune diseases (Lee et al., 1997) and disseminated intravascular coagulation (Oney et al., 2007).

In the oncology field, the most clinically advanced aptamer, and the first anticancer aptamer to be tested in human is a 26-nt G-rich DNA known as AS1411 (formerly, AGRO 100). The mechanism of Guanine Rich Oligonucleotides antiproliferation activity is probably due to the formation of stable quadruplex structure and G-quartets (Gilbert and Feigon, 1999; Dapic et al., 2003; Zhang et al., 2003). These structures interact with a specific cellular protein, nucleolin leading to the inhibition of cell proliferation. In response to stress, nucleolin expression is increased and P53 is activated. Activated P53 stimulates the formation of nucleolin- P53 complex, which participates in transient inhibition of DNA replication and initiation of DNA repair (Daniely et al., 2002).

This guanine rich oligonucleotide (GRO) has the sequence of **5'-GGTGGTGGTGGTTGTGGTGGTGGTGG-3'** and was studied for its effect on human monocytic leukemia cell line U937 (Lei Z., et al., 2011). It was noticed that GRO can inhibit proliferation of U937 cells and induce cell cycle arrest at S phase (Lei Z., et al., 2011).

Fortunately, anti-PDGFRα/β aptamers developed here share this G-motif. This means that these sequences may have a promising anti cancer effect due to the presence of the GGT motif which is responsible for the anti- proliferative activity on cancerous cells.

Epithelial Growth Factor Receptor (EGFR) is a tyrosine kinase receptor, which is up regulated in brain cancer. A phosphorothioate oligonucleotide (PS-ODS) can inhibit the EGFR tyrosine kinase activity in A431cells. The PS-ODS sequence is 5'-GGAGGGTCGCATCGC-3' and acts as sequence dependent tyrosine kinase inhibitor; Sequence deletion and substitution studies revealed that the 5' terminal **GGAGGG** hexameric sequence in the parent compound was essential for the anti tyrosine kinase activity. So, presence of hexameric motif in antisence oligonucleotide resulted in more potent inhibitors of TK activity (Akhtar et al.,2002).

Interestingly; this hexameric motif was found in 80 % (8 from 10) of anti- PDGFRα aptamers, and in 57% (8 from 14) of anti- PDGFRβ aptamers. This demonstrates that our anti-PDGFRα/ anti-PDGFRβ aptamers may act as inhibitors and may be considered as potential therapeutic agents in the future.

6. Conclusion

In conclusion, this study has described the process for selection of anti- PDGFRα/β aptamers. The properties of the selected aptamers make these molecules appealing candidates for further development as enzymatic inhibitors both for laboratory use and as therapeutic agents in the future.

7. Future Works

One the basis of the conclusions of this study, we recommended the following:

- Investigate the inhibitory effect of the selected aptamers on the tyrosine kinase domain in vitro.
- Evaluate of the degree of affinity of the selected aptamers for PDGFR active kinase domain.
- Enhance cellular delivery of the selected aptamer, which is an important functional aspect for therapeutic purposes.
- Investigate the effect of G- quadruplex formed by the selected aptamer on the inhibition of the tyrosine kinase activity.
- Increase nuclease resistance of the selected aptamers.
- Evaluation of the efficiency of inhibition of anti-PDGFR aptamers in vivo.

8. References:

Akhtar S, Dunnion D, Poyner D, Ackroyd J, Bibby M, Double J. (2002), Sequence and chemistry requirements for a novel aptameric oligonucleotide inhibitor of EGF receptor tyrosine kinase activity. **Biochemical Pharmacology**, 63, 2187-2195.

Been MD, Cech TR. (1988), RNA as an RNA polymerase: Net elongation of RNA primer catalyzed by the Tetrahymene ribozyme. **Science**, 238, 1412-1416.

Berens C, Thain A, Schoeder R. (2001), A tetracycline binding RNA aptamer. **Bioorganic and Medical Chemistry**, 9, 2549-2556.

Berezovski M, Musheev M, Drabovich A, Krylov S. (2006), Non Selex Selection of aptamers. **Journal of the American Chemical Society**, 128, 1410-1411.

Bevilacaua MP, Nelson RN. (1993), Selectins. **Journal of Clinical Investigation**, 91, 379-387.

Biesecker G, Dihel L, Enney L, Bendele RA. (1999), Derivation of RNA aptamer inhibitors of human complement C5. **Immunopharmacology**, 42, 219-230.

Bioziau C, Dausse E, Yurchenko L, Toulme J. (1999), DNA aptamers selected against the HIV-1 trans-activation responsive RNA element from RNA-DNA kissing complexes. **Journal of Biological Chemistry**, 274, 12730-12737.

Bock LC, Griffin LC, Latham JA, Vermass EH, Toole JJ. (1992), Selection of single stranded DNA molecules that bind and inhibit human thrombin. **Nature**, 7, 877-880.

Brugert HG, Ruzsics Z, Obermeier S. (2002) Subversion of host defense mechanisms by adenoviruses**. Current Topics in Microbiology and Immunology**, 269, 273-318.

Bruno JG, Kiel JL. (1999), In vitro selection of DNA aptamers to anthrax spores with electrochemiluminescence detection. **Biosensors and Bioelectronics**, 14, 457-464.

Burke HD, Hoffmann DC, Brown A, Hansen M, Pardi A, Gold L. (1997), RNA aptamers to the peptidyl transferase inhibitor chloramphenicol. **Chemical Biology**, 4, 833-843.

Campbell J, Green R. (2006), The myeloproliferative disorders. **New England Journal of medicine**, 355, 2452-2466.

Carroll M, Jones S, Gilliland G, Druker B. (1997), CGP57148, a Tyrosine inhibits the Growth of cells expressing BCR-ABL and TEL-PDGFR fusion proteins. **Blood**, 90, 4947-4952.

Cassiday L, Maher L. (2003), Yeast genetic selections to optimize RNA decoys for transcription factor NF-Kappa B. **National Academy of Sciences**, 100, 3930-3935.

Cerchia L, Duconge F, Pestourie C, Boulay J, Assioni Y, Gombert K, Tavitian B, De Franciscis V, Libri D. (2005), Neutralizing aptamers from whole-cell SELEX inhibit the RET receptor tyrosine kinase. **Public Library of Science Biology**, 3, 697:704.

Cerchia L, Hamm J, Libri D, Taivitian B, Franciscis V. (2002) Nucleic acid aptamers in cancer medicine. **Federation of European Biochemical Societies**, 528, 12-16.

Chalandon J. (2005), Protein tyrosine kinases in hematologic malignancies. **The Hematology Journal**, 9, 950-968.

Cheng C, Dong J, Yao L, Chen A, Jia R, Huan L, Guo J, Zhang Z. (2008), Potent inhibition of human influenza H5N1 virus by oligonucleotide derived by SELEX. **Biochemical and Biophysical Resarch Comunication**, 366, 670-674.

Choi E.W., Nayak L.V., and Bates P.J. (2009), Cancer selective anti proliferative activity is a general property of some G- rich oligodexynucleotides. **Nucleic acid research**, 25, 1-13.

Christopher R, Kelland. Discovery and development of anticancer aptamers. **Molecular Cancer Therapy**. 2006; 12: 2957-2962.

Ciesiolka J, Gorski J, Yarus M. (1995), Selection of RNA domain that binds Zn. **RNA**, 1, 538-550.

Clark, SL, Remcho VT. (2002), Aptamers as analytical reagents. **Electrophoresis**. 23, 1335–1340.

Collin D, HEijenoorte C, Boizian C, Toulem JT, Guittet E.(2000), NMR characterization of a kissing complex formed between the TAR RNA element of HIV-1 and a DNA aptamer. **Nucleic Acids Research**, 28, 3386-3391.

Conrad R, Keranen L, Ellington AD, Newton A. (1994), Isozyme specific inhibition of proteinkinase C by RNA aptamers. **Biological Chemistry**, 269, 32051-32054.

Cox J, Ellington A, Hayhurst A, Hesselberth J, Bayer T, Georgiou G. (2002), Automated selection of aptamers against protein targets translated in vitro: from gene to aptamer. **Nucleic Acids Research,** 3, 1697-1704.

Daniely Y, DimitrovA, D. (2002), Stress-dependent nucleolin mobilization mediated by p53-nucleolin complex formation. **Molecular Cell Biology**, 22, 6014–6022.

David R, Halpin R, Harbury B. (2004), Genetic Manipulation of Combinatorial Chemistry Libraries for small molecule Evolution. **Public Library of Science Biology**, 2, 1022-1030.

Denanda AJ, Coutre SE, Moon MR. (1994), Pilot study of the efficacy of a thrombin inhibitor for use during cardiopulmonary bypass. **Annals of Thoracic Surgery**, 58, 344-350.

Dick LW, McGown LB. (2004), Aptamer-enhanced laser desorption/ionization for affinity mass spectrometry. **Analytical Chemistry**, 76, 3037–3041.

Dova L, Pentheroudakis G, Ntemou A, Fauntzilas G, Pavlidis N. (2007), Targeting c-Kit, PDGFR in cancer of unknown primary: a screening study for molecular markers of benefit. **Journal of Cancer Research and Clinical Oncology**, 7, 432-441.

Drolet D, MoonMcDermott L, Remig T. (1996), An Enzyme Linked Oligonucleotide assay. **Nature Biotechnology**, 14, 1021-1025.

Drolet D, Nelson J, Jucker C, Zack P, Nixon K, Bolin R, Farmer J, Wolf J, Gills C, Bendele R. (2000), Pharmacokinetics and safety of an anti-vascular endothelial growth factor aptamer (NXI838) following injection into the vitreous humor of rhesus monkeys. **Pharmacology Research**, 14, 1503-1510.

Dyke C, Steinhubl S, Kleiman N, Cannon R, Aberle L, lin M, Myles S, Melloni C, Harrington A, Alexander J, Becker R, Rusconi C. (2006), First-in-human experience of an Antidote-controlled Anticoagulant using RNA aptamer technology: A phase 1A pharmacodynamic Evaluation of Drug-Antidote pair for the controlled regulation of factor IXa activity. **Circulation**, 56, 555-583.

Elizabeth H, Benjamin S, Gilliland D. (2005), The small molecule tyrosine kinase inhibitor AMN107 inhibits TEL-PDGFRβ and FIP1L1-PDGFRα in vitro and vivo. **Blood**, 106, 3206-3212.

Ellington AD, Szostak JW. (1990), In vitro selection of RNA molecules that bind specific ligands. **Nature**, 346, 818-822.

Ellington AD, Szostak JW. (1992), Selection in vitro of single-stranded DNA molecules that fold into specific ligand binding structures. **Nature**, 355:850-852.

Eugene W, Shima D, Calias P, Adamis A, Cunningham E, David G. (2006), Pegabtanib, a targeted anti-VEGF aptamer for ocular vascular disease. **Nature Review Drug Discovery**, 5, 123-132.

Eulberg D, Klussmann S. (2003), Spiegelmers: biostable aptamers. **Chemical Biochemistry**, 4, 979-983.

Eytech Study Group. (2003), Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: phase II study results. **Ophthalmology**, 110, 979-986.

Eytech Study Group. (2002), Preclinical and phase 1A clinical evaluation of anti-VEGF pegylated aptamer (EyE001) for the treatment of exudative age-related macular degeneration. **Retina**, 4, 979-983

Famulok M, Harting SJ, Mayer G. (2007), Functional Aptamers and Aptazymes in Biotechnology, Diagnostics, and Therapy. **Chemical Reviews**, 107, 3175-3734.

Famulok M, Jenne A. (1998), Oligonucleotide libraries variation delicate. **Current Opinion in Chemical Biology**, 2, 320-327.

Famulok M. (1994), Molecular recognition of amino acids by RNA aptamer: An L-Citruline binding RNA motif and it is evolution into an L-arginine binder. **Journal of the American Chemical Society,** 116, 1698-1706.

Farokhzad O, Cheng J, Teply B, Sherif I, Jon S, Kantoff P, Richie J, Lauger R. (2006), Targeted nano-particle aptamer bioconjugates for cancer chemotherapy in vivo. **Proceedings of the National Academy of Sciences**, 103, 6315-6320.

Farokzad OC, Jon S, Kademhosseini A, Tran TN, Lavan DA, Langer R. (2004), Nanoparticle-aptamer bioconjugates: A new approach for targeting prostate cancer cells. **Cancer Research**, 64, 7668-7672.

Faulhammer D, Schgfaller E. (2004), Biostable aptamers with antagonistic properties to the nuropeptide nociuptin/orphanin FQ. **RNA**, 10, 516-527.

Flamm C, Fontana W, Hofaker I, Shcuster P. (2000), Elementary step dynamics of RNA folding. **RNA**, 6, 325-338.

Funa K, Uramoto H. (2003), Regulatory mechanisims for the expression and activity of platelet-derived growth factor receptor. **Academy of Behavioral Profiling**, 50, 647-658.

Geiger A, Burgstaller P, Famulok M. (1996), RNA aptamers that bind L-argenine with submicromolar dissociation constants and high enantioselectivity. **Nucleic Acids Research**, 24, 1029-1036.

German I, Buchanan DD, Kennedy RT. (1998), Aptamers as ligands in affinity probe capillary electrophoresis. **Analytical Chemistry,** 70, 4540–4545.

Gilbert, D.E. and Feigon, J. (1999), Multi-stranded DNA structures. **Current Opinion in Structural Biology**, 9, 305–314.

Gilliand GD, Levine LR. (2007), JAK-2 mutation and their relevance to myeloproliferative disease. **Current Opinion in hematology**, 14, 43-47.

Gold L, Polisky B, Uhlenbeck O, Yarus M. (1995), Diversity of oligonucleotide functions. **Annual Reviews of Biochemistry**, 64, 763-797.

Gopinath SC. (2007), Methods developed for SELEX. **Analytical and Bioanalytical Chemistry**, 387, 171-182.

Gragoudas E, Adamis A, Feinsod M, Guyer D. (2004), Pegaptanib for Neovascular age related macular Degeneration. **New England Journal of Medicine**, 351, 2805-2816.

Green L, Jellinek D, Jenison R, Ostmam A, Heldin C, Janjic N. (1996), Inhibitory DNA ligands to platelet-Derived Growth Factor B-chain. **Biochemistry**, 35, 14413-14424.

Griffin LC, Tidmarsh GF, Bock LC, et al. In vivo anticoagulant properties of a novel nucleotide-based thrombin inhibitor and demonstration of regional anticoagulation in extracoorporated circuits. **BLOOD**. 1993; 81: 3217-3226.

Hamula C, Guthrie J, Zhang H, Li X, Le X. (2006), Selection and analytical applications of aptamers. **Trends in Analytical Chemistry**, 25, 681-691.

Hamula CL, Zhang H, Guan LL, Xing-Fang li, Chrisle. (2008), Selection of of aptamers aginst live bacterial cells. **Analytical Chemistry**, 80, 7812-7819.

Heldin C, Osman A, Ronstrand L. (1998), Signal transduction via platelet derived growth factor receptors. **Biochemical and Biophysical Acta**, 1378, 79-113.

Henrik J. (1999), Mechanism of action and in vivo role of platelet- derived growth factor. **Physiological Reviews**, 79, 1283-1316.

Hianik T, Ostatna V, Sonlajtnerova M, German I. (2006), Influence of ionic strength, Ph, and aptamer configuration for binding affinity to thrombin. **Bioelectrochemistry**, 70, 127-133.

Hirao I, Yoshinari S, Yokoyama S, Endo Y, Ellington AD. (1997), In vitro selection of aptamers that bind ribosome-inactivating yoxins. **Nucleic Acids Symposium Series**, 37, 283-284.

Huang D, Cassidy L, Zimmerman M, Maher J, Ghosh G. (2003), Crystal structure of NF- Kb (p50) complexed to a high affinity RNA aptamer. **Proceedings of the National Academy of Sciences**, 86, 7706-7710.

Huang M, Prendergast G, Kleintop L. (2007), RhoB regulates PDGFR trafficking and signaling in vascular smooth muscle cell. **Arteriosclerosis, Thrombosis, and Vascular Biology**, 27, 2597-2605.

Huse M, Kuriyan J. (2002), The conformational plasticity of protein kinases. **Cell**, 109, 275-282.

Jaeger J, Resttle T, Steitz T. (1998), The structure of HIV-1 reverse trascriptase complexed with an RNA pseudoknot inhibitor. **European Molecular Biology Organization**, 17, 4535-4542.

James W. (2007), Aptamers in the virologist tool kit. **Journal of General Virology**, 88, 351-364.

James W. (2000), Aptamers. **Encyclopedia of Analytical Chemistry**, R.A.Myers(ED), 4848-4871.

Jayasena SD. (1999), Aptamers: An emerging class of molecules that rival antibodies in diagnosis**. Clinical Chemistry**, 45, 1628-1650.

Jellinek D, Bell C, Lynott CK, Gillin N, McGee D, Pieken W. (1995), Potent 2'-amino-2'deoxypyremidine RNA inhibitors of basic fibroblast growth factor, **Biochemistry**, 34, 11363-11372.

Jenison RD, Gill SC, Pardi A, Polisky B. (1994), High-resolution molecular discrimination by RNA. **Science**, 263, 1425-1449.

Jenison RD, Jennings SD, Walker DW, Bargatze RF, Parma D. (1998), Oligonucleiotide inhibitors of P-selectine-dependent neutrophil platelet adhesion. **Antisense Nucleic Acid Drug Devel**, 8, 269-279.

Jensen K, Atkinson B, Willis M, Koch T, Gold L. (1995), Using in vitro selection to direct the covalent attachment of human immunodeficiency virus type 1 Rev protein to high affinity RNA ligand. **Proceedings of the National Academy of Sciences**, 92, 12220-12224.

Jhaveri S, Rajendan M, Ellington AD. (2000), In vitro selection of signaling aptamers. **Nature Biotechnology**, 18, 1293-1297.

Jianying Gu and Xun Gu. (2003), Natural history and functional divergence of protein tyrosine kinases. **Gene**, 317, 49-57.

Karn J. Tackling Tat. (1999), **Molecular Biology**, 293, 235-254.

Kelland LR. (2005), Overcoming the immortality of tumor cells by telomere and telomerase based cancer. **European Journal of Cancer**, 41, 971-979.

Klug JS, Famulok M. (1994), All you want to know about SELEX. **Molecular Biology Reports**, 20, 97-107.

Koch T, Smith D, Tabacman E, Zichi D. (2004), Kinetic analysis of site-specific photo aptamer protein cross linking. **Molecular Biology**, 336, 1159-1173.

Kopylov MA, Spiridonova VA. (2000), Combinatorial Chemistry of Nucleic Acids: SELEX. **Molecular Biology**, 97, 349 -370.

Kostich M, English J, Madison V, Gheyas F, Wang L, Qiu P, Greene J. (2002), Human members of the eukaryotic protein kinase family. **Genome Biology**, 3, 1-12.

Kotia RB, Li L, McGown LB. (2000), Separation of non target compounds by DNA aptamers. **Analytical Chemistry**, 72, 827–831.

Kulbachinskiy A. (2007), Methods for selection of aptamers to protein targets. **Biochemistry**, 72, 1505-1518**.**

Kumar P, Machida K, Urvil P, Nishikawa K, Taira K. (1997), Isolation of RNA aptamers specific to the NS3 protein of hepatitis C virus frm a pool of completely random RNA. **Virology**, 237, 270-282.

Kurien BT, Scofield RH. (2002), Extraction of nucleic acid fragments from gels. **Analytical Chemistry**, 302, 1-9.

Lee S.W., B.A. Sullenger. (1997), Isolation of a nuclease resistant decoy RNA that can protect human acetylcholine receptors from myasthenic antibodies, **Nature Biotechnology**, 15, 41-45.

Lei Zhi, Jianwei Zhang, Yujiao Jia, Shilong Shan, Yan Li, Donghai Wang, Min Wang, Qing Rao, Haiyan Xing, Kejing Tang, Zheng Tian, Jianxiang Wang, and Yingchang Mi. (2011), Effect of G-Rich Oligonucleotides on the Proliferation of Leukemia Cells and Its Relationship with p53 Expression. **OLIGONUCLEOTIDES**, 21, (1).

Liu J, Zhou J, Deng X, Ti Z. (), A simple and sensitive dipstick test in serum based on lateral flow separation of aptamer linked nanostructures. **Angewandte Chemie International Edition**, 7955-7959.

Lorsh JR, Szostak JW. (1994), In vitro selection of RNA aptamers specific for cyanocobalamine. **Biochemistry**, 33, 973-982.

Marciniak R, Garcia A, Sharp A. (1990), HIV-I Tat protein Trans activates transcription in vitro. **Cell**, 636, 791- 802.

Marial, T, Ozal V, Sanche Z, Mir M, Katakis I, O'Sullivan C. (2008), Aptamers: molecular tools for analytical applications. **Analytical and Bioanalytical Chemistry**, 390, 989-1007.

Marshall K, Ellington AD. (2000), In vitro selection of RNA aptamers. **Methods in Enzymology**, 318, 193-214.

Maurel CM, Haenni AL. (2005), The RNA world: hypotheses, facts and experimental results. **Lectures in Atrobiology**, 1, 571-594.

Mayer G. The Chemical Biology of Aptamers. (2009), **Gesellschaft Deutscher Chemiker**, 48, 2672-2689.

Mendosa D, Bowser M. (2004), In vitro evolution of functional DNA using capillary electrophoresis. 2004. **Journal of the American Chemical Society**, 126, 20-22.

Mendosa D, Bowser M. (2004), In vitro selection of high affinity DNA ligands for human IgE using capillary electrophoresis. **Analytical Chemistry**, 76, 5387-5392.

Molligan M, Schmid E, Till G. (1997), C5a-dependent up-regulation in vivo of lung vascular P-selectin. **Journal of Immunology**, 158, 1857-1861.

Morgan B. (1995), Physiology and pathophysiology of comlementy: Progress and trends. **Critical Reviews in Clinical Laboratory Sciences**, 32, 185-198.

Murphy MD, Fuller S, Richardosn P, Doyle S. (2003), An improved method for the in vitro evolution of aptamers and applicants in protein detection and purification. **Nucleic Acids Research**, 31, 110-118.

Musheev MU, Krylov SN. (2006), Selection of aptamers by systematic evolution of ligands by exponential enrichment: Addressing the polymerase chain reaction issue. **Analytical and Chemical Acta**, 564, 91-96.

Nimjee SM, Rusconi CP, Sullenger BA. (2005), Aptamers: An emerging class of theraputics. **Annual Reviews of Medicine**, 56, 555-583.

Ortutay C, Valiaho J, Stenberg K, Vihinen M. (2005), A Registry of Disease Causing Mutation in protein kinase domains. **Analytical Chemistry**, 76, 5387-5392.

Osborne ES, Masumura I, Ellington AD. (1997), Nucleic Acid Selection and the Challenge of Combinatorial Chemistry. **Chemical Reviews**, 4, 588-596.

Pagratis N, Bell C, Chang YF, Jennings S, Fitzwater T, Jellinek D, Dany C. (1997), Potent 2-amino-, and 2`-fluro-2`-deoxyribonucleotide RNA inhibitors of keratinocyte growth factor. **Nature Biotechnology**, 15, 68-73.

Paul M. and Mukhopadhyay A. (2004), Tyrosine kinase role and significance in cancer. **Internal Journal of medical sciences**, 1, 101-115.

Pendergrast PS, Marsb HN, Grate D, Healy MJ, Stanton M. (2005), Nucleic acids aptamers for target validation and therapeutic application. **Journal of Biomolecular Techniques**, 16, 224-234.

Phillips JA, Lopez-Colon D, Zhu Z, Xu Y, Tan W. (2008), Application of aptamers in cancer cell biology. **Analytical and Chemical Acta**, 621, 101-108.

Rana TM, Jeang KT. (1999), Biochemical and functional interactions between HIV-1 Tat protein and TAR RNA. **Archives of Biochemistry and Biophysics**, 365, 175-184.

Reilly J.T. (2002), Class III receptor tyrosine kinases: role in leukomognesis. **British Journal of Hematology**, 116, 744-757.

Rusconi CP, Roberts JD. (2004), Antidote-mediated control of an anti-coagulant aptamer in vivo. **Nature Biotechnology**, 22, 1423-1428.

Sampson T. (2003), Aptamers and SELEX: the technology. **World Patent Information**, 25, 123-129.

Santalucia JJ, Hicks D. (2004), The thermodynamics of DNA structural motifs. **Annual Reviews of Biophysics & Biomolecular Structure**, 33, 415-440.

Santalucia JJ. (1998), A unified view of polymer, dumbbell and oligonucleotide DNA nearst-neighbor thermodynamics. **Proceedings of the National Academy of Sciences**, 95, 1460-1465.

Saran D, Frank J, Burke D. The tyranny of adenosine recognition among RNA aptamers to Coenzyme A. (2003), **BioMed Central Evolutionary Biology**, 3, 555-583.

Sassonfar M, Szostak JW. (1993), An RNA motif that binds ATP. **Nature**, 364, 550-553.

Scheijen B, Griffin DJ. (2002), Tyrosine kinase oncogenes in normal hematopoiesis and hematological disease. **Oncogene**, 21, 3314-3333.

Schmidt SK, Borkowski S, Kurreck J, Stephenes AW, Bald R, Hecht M, Dinkelborg L, Erdmann AV. (2004), Application of locked nucleic acids to improve aptamer in vivo stability and targeting function. **Nucleic Acids Research**, 32, 5757-5765.

Seiwert S, Naheini T, Aigner S, Ahn N, Uhlenbleck O. (2000), RNA aptamers as pathway- specific kinase inhibitors. **Chemical Biology**, 7, 833-843.

Shangguan D, Li Y, Tang Z, Cau Z, Chen H, Mallikaratchy P, Setan K, Yang C, Tan W. (2006), Aptamers evolved from line cells as effective molecular probes for cancer study. **Proceedings of the National Academy of Sciences**, 103, 11838-11843.

Shimada T, Fujita N, Meda M, Ishihama A. (2005), Systematic search for the Cra binding promoters using genomic SELEX system. **Genes Cells**, 10, 907-918.

Simonsson T, Pecinka P, Kubistan M. (1998), DNA tetraplex formation in the central region of c-myc. **Nucleic Acids Research**, 26, 1167-1172.

Simonsson T. (2001), G-quadraplex DNA structures- variations. On a theme. **Nucleic Acids Research**, 33, 2901-2907.

Singer C, Hudelist G, Lamm W, Muller R, Gzerwenka K, Kubista E. (2004), Expression of tyrosine kinases in human malignancies as potential targets for kinase specific inhibitors. **Endocrine Related Cancer**, 11, 861-869.

Stojanovic MN, Landry DW. (2002), Aptamer-based colorimetric probe for cocaine. **Journal of the American Chemical Society**, 124, 9678–9679.

Stolenburg R, Reinemann C, Strenlitiz B. (2007), SELEX – A (r) evolutionary method to generate high affinity nucleic acid ligands. **Biomolecular Engineering**, 24, 381-403.

Stover J. (2005), The small molecule tyrosine kinase inhibitor AMN107 inhibits TEL-PDGFR β and FIP1I1-PDGFR α in vitro and in vivo. **Nucleic Acids Research**, 33, 2901-2907.

Swick B, Ravdel L, Fitzptrick J, Robinson W. (2008), PDGFR α mutational status and immunohistochemical expression in merkel cell carcinoma: implication for treatment with imatinib mesylate. **Journal of Cutaneous pathology**, 35, 197-208.

Tamborini E, Miselli F, Negri T, Dagrada G, Pastore E, Pilotti S. (2006), Molecular and Biochemical Analyses of Platelet Derived Growth Factor Receptor (PDGFR) B ,

PDGFRA, and kit receptors in Chordomas. **Clinical Cancer Research**, 23, 6920-6928.

Till HJ, HubBard RS. (2004), Protein tyrosine kinase structure and function. **Annual Reviews of Biochemistry**, 69, 373-398.

Tombelli S, Minunni M, Mascini M. (2005), Analytical applications of aptamers. **Biosensors and Bioelectronics**, 20, 2424–2434.

Tuerk C, Gold L. (1990), Systematic Evolution of Ligands by Exponential Enrichment: RNA ligands to Bacteriophage T4 DNA polymerase. **Science**, 249, 505-510.

Turek C, Macdougal S, Gold L, (1992), RNA pseudoknots that inhibit human immunodeficiency-virus type 1 reverse transcriptase. **Proceedings of the National Academy of Sciences**, 89, 6988-6992.

Uehara. (2003), Eeffect of blocking PDGFR signaling in a mouse model of experimental prostate cancer bone metastases. **Journal of the National Cancer Institute**, 95, 558-570.

Ullrich H, Alves M.J.M and Colli W. (2001), RNA and DNA aptamers as potential tools to prevent cell adhesion in disease. **Journal of Medical and Biological Research**, 34, 295-300.

Ulrich H, Magdesian M, Alves M, Colli W. (2002), In vitro selection of RNA aptamers that bind to cell adhesion receptors of trypanosome cruzi and inhibit cell invasion**. Journal of Biological Chemistry**, 277, 20756-20762.

Ulrich H, Martin A, Pesquero J. (2004), RNA and DNA aptamers in cytomics analysis. **Cytometry**, 59, 220-231.

Ulrich H, Wrenger C. (2009), Disease specific Biomarker Discovery by aptamers. **Cytometry**, 75, 727-733.

Valian S, Khazaei M. (2007), Medical applications of aptamers. **Royal Photographic Society**, 2, 59-66.

Vater A, Klussmann S. (2003), Toward third generation aptamers: spiegelmers and their therapeutic prospects. **Current Opinion in Drug Discovery & Development**, 6, 253-261.

Verdine G. (1996), The combinatorial chemistry of nature. **Nature**, 384, 11-13.

Vinores SA. (2003), Technology evolution: Pegaptanib. **Nucleic Acid Research**, 33, 2901-2907.

Wallis MG, Schroeder R. (1997), The binding of antibiotics to RNA. **Progress in Biophysics and Molecular Biology**, 67, 141-154.

Wange W, Yun J. (2009), Progress in aptamer screening methods. **Chinese Journal of Analytical Chemistry**, 37, 454-460.

Watson JD, Crick FH. (1953), Molecular Structure of Nucleic Acids. **Nature**, No 4356:1.

Watson SR, Chang YF, O'Connell D, Weigand L, Ringquist S, Parma DH. (2000), Anti-L-Selectin aptamers: binding characteristics, pharmacokinetic parameters, and activity against an intravascular target in vivo. **Antisense Nucleic Acid Drug Development**, 10, 63-75.

Weigand T, Williams P, Dreskin S, Jouvin M, Kinet J, Tasset D. (1996), High affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor 1. **Nature**, 157, 221-230.

Wen J, Gray D. (2005), Selection of genomic sequences that bind tightly to Ff gene 5 protein: primer free genomic selex. **Nucleic Acid Research,** 32, 2-10.

White R, Rusconi C, Scardino E, Wolberg A, Lawson J, Hoffman M. (2001), Generation of species cross- reactive aptamers using "Toggle" SELEX. **Molecular Therapy**, 4, 567-573**.**

Wilson SD, Szostak WJ. (1999), In vitro selection of functional nucleic acids. **Annual Reviews of Biochemistry**; 68, 611-647.

Yamamoto R, Kumar PK. (2000), Molecular beacon aptamer fluoresces in the presence of Tat proteins of HIV-1. **Silence**, 5, 389-396.

Yang Q, Goldstein M, Mei HY, Enylke DR. (1998), DNA ligands that bind tightly and selectivity to cellobiose. **Proceedings of the National Academy of Sciences**, 95, 5642-4567.

Yang Y, Yang D, Schluesener H, Zhang Z. (2007), Advances in SELEX and applications of aptamers in central nervous system. **Biomolecular Engineering**, 24, 583-592.

Yarden Y. and Ullrich A. (1988), Growth factor receptor tyrosine kinase, **Annual Reviews of Biochemistry**, 57, 443-478.

Zhou J, Rossi J. (2010), Aptamer targeted cell specific RNA interference. **Silence**, 1, 1-10.

Zuker, M. (2003), Mfold web server for nucleic acid folding and hypredization prediction. **Nucleic Acids Research**, 31, 3406-3415.

انتقاء سلاسل من الحمض النووي المضاد لمستقبل عامل النمو المشتق من الصفائح الدموية بطريقة التطوير النظامي لرابطات بالتكثير المتضاعف

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الأستاذ الدكتور عــزمـــي مــحافــظة

مـــلـــخص

الأبتامرز عبارة عن سلاسل من الحمض النووي يتم انتقاؤها عن طريق عملية التطوير النظامي للرابطات بالتكثير المتضاعف (SELEX)؛ و هذه السلاسل لديها القدرة على تكوين أشكال ثلاثية الأبعاد لترتبط مع جزيئات البروتين المختلفة بتقارب عالي و نوعية مماثلة للأجسام المضادة. و لهذه الجزيئات القدرة على تعديل نشاط البروتينات المستهدفة من خلال الربط بمناطق معينة. أحد هذه البروتينات هو المستقبل لعامل النمو المشتق من الصفائح الدموية (PDGFR) و المعروف بدوره الرئيسي في العديد من السرطانات المختلفة و التشوهات الجينية المكتسبة.

أهداف الدراسة: تهدف هذه الدراسة إلى تطوير سلاسل من الحمض النووي (الأبتامرز) قادرة على الربط مع المستقبل لعامل النمو المشتق من الصفائح الدموية (PDGFR)؛ و دراسة هذه السلاسل و مقارنتها مع سلاسل أخرى موجهه لبروتينات مختلفة لتوقع عملها كمثبط لنشاط البروتينات المرتبطة بها.

الأساليب: تم ربط البروتينات مع مصفوفة من الأبتامرز طولها 90 قاعدة نيتروجينية تحتوي على 45 قاعدة نيتروجينية عشوائية في المنطقة الوسطى ، بعد عملية الارتباط يتم عزل سلاسل الحمض النووي الرابطة و تضخيمها عن طريق تفاعل البلمرة المتسلسل (Polymerase Chain Reaction) ثم يتم تكرار هذه العملية لتحديد السلاسل الموجهة للبروتينات ذات تقارب و خصوصية عالية.

النتائج: بعد الجولة العاشرة؛ تم استنساخ سلاسل الحمض النووي موجهة لمستقبل عامل النمو المشتق من الصفائح الدموية و معرفة محتوياتها من القواعد النيتروجينية؛ و بعد التحليل تم التوصل إلى 24 سلسة من الحمض النووي موجهة للبروتين و تم دراسة هذه السلاسل و مقارنتها بسلاسل أخرى مختارة معروفة بالفعل أنها تثبط عمل البروتينات

الاستنتاجات: وصفت هذه الدراسة طريقة تطوير سلاسل من الحمض النووي موجهة لمستقبل عامل النمو المشتق من الصفائح الدموية و خصائص هذه السلاسل التي قد تجعلها تلعب دورا مهما و أساسيا في علاج العديد من الأورام السرطانية في المستقبل.